BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

Claims 21-40 are pending in the application. Claims 30, 32-34 and 37-40 are withdrawn as being drawn to non-elected inventions. Claims 21 and 31 have been amended. Claims 21-29, 31, 35, and 36 are under active consideration.

To expedite prosecution, claim 21 has been amended to recite that the claimed biologically active fragments have FHL3 activity, and claim 31 has been amended to recite that the claimed polynucleotide variants encode a polypeptide having FHL3 activity. Support for the amendment of claims 21 and 31 can be found in the specification, for example, at pages 1-2, which describe LIM proteins, page 12, lines 14-23, which points out the presence of four LIM domains in LDPH, and at pages 42-45, which describe assays for LDPH activity. In addition, a recent Blast analysis shows that SEQ ID NO:1 is 100% identical to the human four and a half LIM domain 3 (FHL3) protein (g30582943). See the BLAST analysis attached at Exhibit A and the articles of Turner et al. (2003) J. Biol. Chem. 278:12786-12795 and Coghill et al. (2003) J. Biol. Chem. 278:24139-24152) describing the activity of FHL3. By this amendment, Applicants expressly do not disclaim equivalents of the invention which could include polypeptides having biological activities in addition to the recited FHL3 activity or polynucleotides encoding such polypeptides. In addition, claim 21 has been amended to recite that the claimed immunogenic fragments consist of at least 5 contiguous amino acid residues of the amino acid sequence of SEQ ID NO:1, wherein said fragment generates and antibody that specifically binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:1. Support for this amendment to claim 21 can be found in the specification, for example, at page 5, lines 18-21, and in Example XII at page 44. These amendments further clarify the intended subject matter of the claimed invention and address the rejections under 35 U.S.C. § 112, first and second paragraphs, 35 U.S.C. § 102 and § 103. Entry of these amendments is respectfully requested.

Applicants reserve the right to prosecute non-elected subject matter in subsequent divisional applications.

Comments Regarding Restriction Requirement

Applicants affirm the election with traverse of Group II, which corresponds to claims 23-29, and 31 drawn to polynucleotides. Applicants thank the Examiner for rejoining claims 21, 22, 35, and 36, drawn to polypeptides, with the claims of Group II.

Rejoinder

Applicants reiterate their request that claims 32-34, 39, and 40, drawn to methods of using the polynucleotides, be rejoined, and in addition, request that claims 37 and 38, drawn to methods of using the polypeptides, be rejoined per the Commissioner's Notice in the Official Gazette of March 26, 1996, entitled "Guidance on Treatment of Product and Process Claims in light of *In re Ochiai, In re Brouwer* and 35 U.S.C. § 103(b)" which sets forth the rules, upon allowance of product claims, for rejoinder of process claims covering the same scope of products. Applicants request that claims 32-34, 39, and 40 be rejoined and examined upon allowance of any of the claims drawn to the polynucleotides of Group II and that claims 37 and 38 be rejoined and examined upon allowance of any of the claims drawn to the polypeptides.

Objection to the Specification

An abstract was added to the specification to comply with 37 CFR 1.72(b).

Written description rejections under 35 U.S.C. § 112, first paragraph

Claims 21, 23, 26-28, and 35 have been rejected under the first paragraph of 35 U.S.C. 112 for alleged lack of an adequate written description. Applicants note that the polypeptide variants of claim 21 have been canceled; therefore, the rejection on this basis is moot. Although not conceding to the propriety of the rejection, to expedite prosecution, Applicants have amended claim 21 to recite that the claimed biologically active fragments have FHL3 activity, and claim 31 to recite that the claimed polynucleotide variants encode a polypeptide having FHL3 activity. Applicants respectfully traverse the rejection.

The requirements necessary to fulfill the written description requirement of 35 U.S.C. 112, first paragraph, are well established by case law.

> . . . the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the "written description" inquiry, *whatever is now claimed. Vas-Cath, Inc. v. Mahurkar*, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991)

Attention is also drawn to the Patent and Trademark Office's own "Guidelines for Examination of Patent Applications Under the 35 U.S.C. Sec. 112, para. 1", published January 5, 2001, which provide that :

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. (footnotes omitted.)

Thus, the written description standard is fulfilled by both what is specifically disclosed and what is conventional or well known to one skilled in the art.

SEQ ID NO:1 and SEQ ID NO:2 are specifically disclosed in the application (see, for example, page 2, lines 12-13 and lines 26-27). Variants of SEQ ID NO:1 and SEQ ID NO:2 are described, for example, at page 12, lines 30-33 and page 13, lines 3-10. Incyte clones in which the nucleic acids encoding the human LIM domain protein homolog (LDPH) were first identified and libraries from which those clones were isolated are described, for example, at page 12, lines 6-12 of the Specification. Chemical and structural features of SEQ ID NO:1 are described, for example, on page 12, lines 13-29. Given SEQ ID NO:2, one of ordinary skill in the art would recognize a polynucleotide comprising a naturally occurring polynucleotide sequence at least 90% identical to the polynucleotide sequence of SEQ ID NO:2, said polynucleotide encoding a polypeptide having FHL3 activity. Accordingly, the Specification provides an adequate written description of the recited polypeptide and polynucleotide sequences.

The Office Action has further asserted that the claims are not supported by an adequate written description because "no disclosure, beyond the mere mention of variants is made in the specification. This is insufficient to support the generic claims as provided y the Interim Written description Guidelines published in the June 15, 1998 Federal Register at Volume 63, Number 114, pages 32639-32645" (Office Action, page 5).

Such a position is believed to present a misapplication of the law.

1. The present claims specifically define the claimed genus through the recitation of chemical structure

Court cases in which "DNA claims" have been at issue (which are hence relevant to claims to proteins encoded by the DNA) commonly emphasize that the recitation of structural features or chemical or physical properties are important factors to consider in a written description analysis of such claims. For example, in *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993), the court stated that:

> If a conception of a DNA requires a precise definition, such as by structure, formula, chemical name or physical properties, as we have held, then a description also requires that degree of specificity.

In a number of instances in which claims to DNA have been found invalid, the courts have noted that the claims attempted to define the claimed DNA in terms of functional characteristics without any reference to structural features. As set forth by the court in *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997):

> In claims to genetic material, however, a generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA," without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function.

Thus, the mere recitation of functional characteristics of a DNA, without the definition of structural features, has been a common basis by which courts have found invalid claims to DNA. For example, in *Lilly*, 43 USPQ2d at 1407, the court found invalid for violation of the written description requirement the following claim of U.S. Patent No. 4,652,525:

> 1. A recombinant plasmid replicable in procaryotic host containing within its nucleotide sequence a subsequence having the structure of the reverse transcript of an mRNA of a vertebrate, which mRNA encodes insulin.

In *Fiers*, 25 USPQ2d at 1603, the parties were in an interference involving the following count:

A DNA which consists essentially of a DNA which codes for a human fibroblast interferon-beta polypeptide.

Party Revel in the *Fiers* case argued that its foreign priority application contained an adequate written description of the DNA of the count because that application mentioned a potential method for isolating the DNA. The Revel priority application, however, did not have a description of any particular DNA structure corresponding to the DNA of the count. The court therefore found that the Revel priority application lacked an adequate written description of the subject matter of the count.

Thus, in *Lilly* and *Fiers*, nucleic acids were defined on the basis of functional characteristics and were found not to comply with the written description requirement of 35 U.S.C. §112; *i.e.*, "an mRNA of a vertebrate, which mRNA encodes insulin" in *Lilly*, and "DNA which codes for a human fibroblast interferon-beta polypeptide" in *Fiers*. In contrast to the situation in *Lilly* and *Fiers*, the claims at issue in the present application define polynucleotides and polypeptides in terms of chemical structure, rather than functional characteristics. For example, the "variant language" of independent claim 31 recites chemical structure to define the claimed genus:

31. An isolated polynucleotide selected from the group consisting of:...
b) a polynucleotide comprising a naturally occurring polynucleotide sequence at least 90% identical to the polynucleotide sequence of SEQ ID NO:2, said polynucleotide encoding a polypeptide having FHL3 activity...

From the above it should be apparent that the claims of the subject application are fundamentally different from those found invalid in *Lilly* and *Fiers*. The subject matter of the present claims is defined in terms of the chemical structure of SEQ ID NO:1 and SEQ ID NO:2. In the present case, there is no reliance merely on a description of functional characteristics of the polynucleotides or polypeptides recited by the claims. The polynucleotides and polypeptides defined in the claims of the present application recite structural features, and cases such as *Lilly* and *Fiers* stress that the recitation of structure is an important factor to consider in a written description analysis of claims of this type. By failing to base its written description inquiry "on whatever is now claimed," the Office Action failed to provide an appropriate analysis of the

present claims and how they differ from those found not to satisfy the written description requirement in *Lilly* and *Fiers*

2. The present claims do not define a genus which is "highly variant"

Furthermore, the claims at issue do not describe a genus which could be characterized as "highly variant." Available evidence illustrates that the claimed genus is of narrow scope.

In support of this assertion, the Examiner's attention is directed to the enclosed reference by Brenner et al. ("Assessing sequence comparison methods with reliable structurally identified distant evolutionary relationships," Proc. Natl. Acad. Sci. USA (1998) 95:6073-6078). Through exhaustive analysis of a data set of proteins with known structural and functional relationships and with <90% overall sequence identity, Brenner et al. have determined that 30% identity is a reliable threshold for establishing evolutionary homology between two sequences aligned over at least 150 residues. (Brenner et al., pages 6073 and 6076.) Furthermore, local identity is particularly important in this case for assessing the significance of the alignments, as Brenner et al. further report that ≥40% identity over at least 70 residues is reliable in signifying homology between proteins. (Brenner et al., page 6076.)

The "variant language" of the present claims recites, for example, polynucleotides encoding "a naturally occurring polynucleotide sequence at least 90% identical to the polynucleotide sequence of SEQ ID NO:2, said polynucleotide encoding a polypeptide having FHL3 activity." This variation is far less than that of all potential polynucleotides encoding LIM domain proteins.

3. The state of the art at the time of the present invention is further advanced than at the time of the *Lilly* and *Fiers* applications

In the *Lilly* case, claims of U.S. Patent No. 4,652,525 were found invalid for failing to comply with the written description requirement of 35 U.S.C. §112. The '525 patent claimed the benefit of priority of two applications, Application Serial No. 801,343 filed May 27, 1977, and Application Serial No. 805,023 filed June 9, 1977. In the *Fiers* case, party Revel claimed the benefit of priority of an Israeli application filed on November 21, 1979. Thus, the written

description inquiry in those case was based on the state of the art at essentially at the "dark ages" of recombinant DNA technology.

The present application has a priority date of September 3, 1998. Much has happened in the development of recombinant DNA technology in the 21 or more years from the time of filing of the applications involved in *Lilly* and *Fiers* and the present application. For example, the technique of polymerase chain reaction (PCR) was invented. Highly efficient cloning and DNA sequencing technology has been developed. Large databases of protein and nucleotide sequences have been compiled. Much of the raw material of the human and other genomes has been sequenced. With these remarkable advances one of skill in the art would recognize that, given the sequence information of SEQ ID NO:1 and SEQ ID NO:2, and the additional extensive detail provided by the subject application, the present inventors were in possession of the claimed polynucleotide variants at the time of filing of this application.

4. Summary

The Office Action failed to base its written description inquiry "on whatever is now claimed." Consequently, the Action did not provide an appropriate analysis of the present claims and how they differ from those found not to satisfy the written description requirement in cases such as *Lilly* and *Fiers*. In particular, the claims of the subject application are fundamentally different from those found invalid in *Lilly* and *Fiers*. The subject matter of the present claims is defined in terms of the chemical structure of SEQ ID NO:1 or SEQ ID NO:2 The courts have stressed that structural features are important factors to consider in a written description analysis of claims to nucleic acids and proteins. In addition, the genus of polynucleotides and polypeptides defined by the present claims is adequately described, as evidenced by Brenner et al and consideration of the claims of the '740 patent involved in *Lilly*. Furthermore, there have been remarkable advances in the state of the art since the *Lilly* and *Fiers* cases, and these advances were given no consideration whatsoever in the position set forth by the Office Action.

Enablement rejections under 35 U.S.C. § 112, first paragraph

Claims 21, 23, 26-28, and 35 are rejected for allegedly failing to meet the requirements of 35 U.S.C. § 112, first paragraph, on the grounds that the Specification does not provide an enabling disclosure commensurate in scope with the claims (Office Action, page 5). In particular, the Examiner alleges that "the specification, while being enabling for an isolated polynucleotide encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:1 and the polypeptide encoded thereby, does not reasonably provide enablement for an isolated a [sic] naturally occurring polynucleotide at least 90% identical to SEQ ID NO:2 or an isolated a [sic] naturally occurring polypeptide at least 90% identical to SEQ ID NO:1" (Office Action, page 5). Applicants traverse the rejection for at least the following reasons.

As set forth in *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971):

> The first paragraph of § 112 requires nothing more than **objective enablement**. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.
>
> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

Applicants submit that the disclosure amply enables the claimed invention. Given the sequence of SEQ ID NO:2, one of ordinary skill in the art could readily identify a polynucleotide comprising a naturally occurring polynucleotide sequence at least 90% identical to a polynucleotide sequence of SEQ ID NO:2, using well known methods of sequence analysis without any undue experimentation. For example, the identification of relevant polynucleotides could be performed by hybridization and/or PCR techniques that were well-known to those skilled in the art at the time the subject application was filed and/or described throughout the Specification of the instant application. See, e.g., page 14, lines 1-33; page 20, line 29 through page 30, line 7; and Example VI at page 40. Thus, one skilled in the art need not make and test

vast numbers of polynucleotides. Instead, one skilled in the art need only screen a cDNA library or use appropriate PCR conditions to identify relevant polynucleotides that already exist in nature. The skilled artisan would also know how to use the claimed polynucleotides, for example in expression profiling, disease diagnosis, or detection of related sequences as discussed above. The specification also describes the expression vectors into which the claimed variants and fragments could be inserted, and the construction of fusion proteins (pages 17-21 and Example IX at pages 41-42).

Applicants respectfully point out that the claims of the instant application are drawn to **naturally occurring** variants. Thus it is not necessary to screen every conceivable variant which might be made using recombinant methods, as all that is claimed are those variant sequences which are found in nature. Through the process of natural selection, nature will have determined the appropriate sequences.

Further, the Examiner requires working examples (Office Action, page 15). There is no such requirement under the law to provide "working examples." As set forth in *In re Borkowski,* 164 USPQ 642, 645 (CCPA 1970) (footnote omitted):

> However, as we have stated in a number of opinions, a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation.

See also M.P.E.P. 2164.02 as follows:

> Compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed. An example may be "working" or "prophetic"... A prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved.

Thus, there is no requirement under the law to provide "working examples" of what is claimed. Rather, one looks to whether the specification provides a description of how to make what is claimed. The present specification provides the requisite description.

Contrary to the standard set forth in *Marzocchi* and *Borkowski*, the Examiner has failed to provide any ***reasons*** why one would doubt that the guidance provided by the present specification would enable one to make and use the recited polynucleotides and polypeptides. Hence, a *prima facie* case for non-enablement has not been established. For at least the above

reasons, withdrawal of the enablement rejections under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 21, 23, 26-28, 31, and 35 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite (Office Action, page 7). In particular, it is alleged that in claims 21 and 31, the recitation of the term "naturally occurring" is indefinite because "[i]t is unclear whether this term imposes a required limitation on the claim, such that it only encompasses, for example, nucleic acid molecules amplified from cDNA or all nucleic acid molecules that encode the polypeptide." In claim 23, the term, "biologically active" is allegedly indefinite because "it is unclear what the metes and bounds of this limitation are because a single amino acid encompasses a 'biologically active fragment' and meets the limitations of this claim." In claim 21, the term, "immunogenic fragment" is allegedly indefinite because "it is unclear what the metes and bounds of this term are." Applicants traverse the rejections on at least the following grounds.

The term "naturally occurring" is a well-known term in the art which Applicants intended to be used in such context. As such, no further definition of the term is necessary (MPEP 2163 IIA3(a)):

> What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94. If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met. See, e.g., *Vas-Cath*, 935 F.2d at 1563, 19 USPQ2d at 1116; *Martin v. Johnson*, 454 F.2d 746, 751, 172 USPQ 391, 395 (CCPA 1972) (stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient").

One of ordinary skill in the art would recognize that a "naturally occurring" sequence as recited in claims 21 and 31 is one which occurs in nature. Through the process of natural selection, nature will have determined the appropriate sequences. The use of the term "naturally occurring" distinguishes a sequence that occurs in nature from synthetic or engineered sequences that do not. One of skill in the art would understand the meaning of the term "naturally occurring" within the context of the claims. Applicants are claiming those polynucleotides and

polypeptides comprising sequences that already exist in nature. Applicants wish to clarify that the claim language does not preclude making such sequences synthetically. Claims 21 and 31 are composition of matter claims that do not refer to methods of making the polynucleotides and polypeptides. Applicants are claiming a subgenus of variants of SEQ ID NO:1 and SEQ ID NO:2, those comprising naturally occurring sequences, as opposed to all variant sequences that can be made synthetically.

To expedite prosecution, claim 21 has been amended to recite that the claimed biologically active fragments have FHL3 activity.

To expedite prosecution, claim 21 has been amended to recite that the claimed immunogenic fragments consist of at least 5 contiguous amino acid residues of the amino acid sequence of SEQ ID NO:1 and generate an antibody that specifically binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:1.

These amendments further clarify the intended scope of the claims. For at least the above reasons, withdrawal of the rejections under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Rejections under 35 U.S.C. § 102(b)

Claims 21 and 23 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Morgan et al. (1996) Biochem. Biophys. Res. Commun. 225: 632-638 on the grounds that the reference discloses a cDNA encoding a LIM-protein expressed in skeletal muscle, SLIM" and that "[a] biologically active fragment of the polypeptide of the reference, would potentially be any amino acid. Therefore, the cDNA and polypeptide disclosed in the reference meets the limitations of claims 21, 23" (Office Action, page 8).

Applicants submit that the reference does not read on claim 21, as currently amended, which now recites "a biologically active fragment of a polypeptide having the amino acid sequence of SEQ ID NO:1, said fragment having FHL3 activity." Therefore, the reference does not anticipate the claimed biologically active fragments, nor polynucleotides encoding them, and Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 102(b).

Rejections under 35 U.S.C. § 103

Claims 26-28, and 35 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Morgan et al. (1996) Biochem. Biophys. Res. Commun. 225: 632-638 on the grounds that:

> it would have been prima facie obvious to one of ordinary skill in the art at the time the instant invention was made to place the polynucleotide encoding the SLIM protein, in an expression vector and host cell which expresses the protein encoded thereby, and recovering the recombinant protein produced to study the biochemical properties of the protein.... Furthermore, it would have been obvious to one of ordinary skill in the art at the time that the invention was made, to merely admix a carrier with a protein i.e. the protein, and obtaining such does not render the resulting composition patentable if it would have been obvious to formulate the protein with a pharmaceutically acceptable carrier relative to its art intended use (Office Action, pages 8-9).

To support an obviousness rejection under 35 U.S.C. § 103, "all the claim limitations must be taught or suggested by the prior art." M.P.E.P. § 2143.03. In addition, "the reference teachings must somehow be modified in order to meet the claims. The modification must be one which would have been obvious to one of ordinary skill in the art at the time the invention was made." M.P.E.P. § 706.02.

Although not acquiescing in the reasons for this rejection, Applicants have amended claim 21 to add a functional limitation for the claimed biologically active fragments. The reference of Morgan et al. does not disclose the claimed polypeptide and polynucleotide sequences of claims 21 and 23; therefore, the rejection of dependent claims 26-28 and 35 under 35 U.S.C. § 103(a) is improper because the reference does not teach or suggest all the limitations of these claims. Withdrawal of the rejection under 35 U.S.C. § 103(a) is respectfully requested.

CONCLUSION

In light of the above amendments and remarks, Applicants submit that the present application is fully in condition for allowance, and request that the Examiner withdraw the outstanding objections/rejections. Early notice to that effect is earnestly solicited.

If the Examiner contemplates other action, or if a telephone conference would expedite allowance of the claims, Applicants invite the Examiner to contact the undersigned at the number listed below.

Please charge Deposit Account No. **09-0108** in the amount of **$110.00** as set forth in the enclosed fee transmittal letter. If the USPTO determines that an additional fee is necessary, please charge any required fee to Deposit Account No. 09-0108.

Respectfully submitted,

INCYTE CORPORATION

Date: February 13, 2004

for James M. Verna, Ph.D. Reg No 41,113

Reg. No. 33,287

Direct Dial Telephone: (650) 845 -5415

Date: February 13, 2004

Jenny Buchbinder

Reg. No. 48,588

Direct Dial Telephone: (650) 843-7212

Customer No.: 27904
3160 Porter Drive
Palo Alto, California 94304
Phone: (650) 855-0555
Fax: (650) 849-8886

Enclosures:

1. Turner et al. J. Biol. Chem. 278:12786-12795 (2003).
2. Coghill et al. J. Biol. Chem. 278:24139-24152 (2003).
3. Exhibit A

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 15, Issue of April 11, pp. 12786–12795, 2003
Printed in U.S.A.

The LIM Protein FHL3 Binds Basic Krüppel-like Factor/Krüppel-like Factor 3 and Its Co-repressor C-terminal-binding Protein 2*

Received for publication, January 19, 2003
Published, JBC Papers in Press, January 29, 2003, DOI 10.1074/jbc.M300587200

Jeremy Turner, Hannah Nicholas, David Bishop, Jacqueline M. Matthews, and Merlin Crossley‡

From the School of Molecular and Microbial Biosciences, G08, University of Sydney, New South Wales 2006, Australia

The ability of DNA-binding transcription factors to recruit specific cofactors is central to the mechanism by which they regulate gene expression. BKLF/KLF3, a member of the Krüppel-like factor family of zinc finger proteins, is a potent transcriptional repressor that recruits a CtBP co-repressor. We show here that BKLF also recruits the four and a half LIM domain protein FHL3. Different but closely linked regions of BKLF mediate contact with CtBP2 and FHL3. We present evidence that CtBP2 also interacts with FHL3 and demonstrate that the three proteins co-elute in gel filtration experiments. CtBP and FHL proteins have been implicated in both nuclear and cytoplasmic functions, but expression of BKLF promotes the nuclear accumulation of both FHL3 and CtBP2. FHL proteins have been shown to act predominantly as co-activators of transcription. However, we find FHL3 can repress transcription. We suggest that LIM proteins like FHL3 are important in assembling specific repression or activation complexes, depending on conditions such as cofactor availability and promoter context.

The Sp/Krüppel-like factor (KLF)[1] family of mammalian DNA-binding proteins consists of the Sp1-related proteins (Sp1–6) and a subfamily termed Krüppel-like Factors (KLF1–17), consisting of erythroid Krüppel-like factor (EKLF/KLF1), lung Krüppel-like factor/KLF2, basic Krüppel-like factor/KLF3, and others (1–4). The Sp/KLF proteins play diverse roles in regulating gene expression during development. For example, EKLF/KLF1 is important for β-globin gene expression (5, 6), and LKLF/KLF2 plays critical roles in lung development, T cell maturation, and in endothelial cells (7–9). Sp/KLF proteins contain a characteristic DNA binding domain (DBD) at or near their C terminus that consists of three Krüppel-type Cys_2His_2 zinc fingers that bind GC and CACCC boxes in regulatory elements of genes. Different members of the family exhibit similar DNA-binding specificity, but in general the Sp1-like subgroup has a higher affinity for GC boxes and the KLF subgroup proteins bind more strongly to CACCC sequences.

Once bound to DNA the proteins are thought to regulate transcription by recruiting co-regulatory molecules. The N-terminal domains of different Sp/KLF family members exhibit little or no homology, and differential cofactor recruitment by these divergent domains may in part explain why some members behave as activators, whereas others act as repressors. The situation is complex as other regions of the proteins, such as the zinc finger domain also mediate protein-protein interactions, and individual proteins may recruit both co-activators and co-repressors. For instance, EKLF/KLF1 has been shown to activate transcription by recruiting the histone acetylase proteins p300/CBP and P/CAF through its N terminus and the chromatin remodeling complex E·RC1 through its zinc finger domain (10). The zinc finger domain can also recruit a histone deacetylase complex to silence gene expression (11).

Other KLFs that can repress transcription include BKLF/KLF3, KLF8, and Ap-2rep/KLF12. These proteins recruit co-repressors of the C-terminal-binding protein (CtBP) family (12–15). Their interaction with CtBP is mediated through a short amino acid motif, of the form Pro-*X*-Asp-Leu-Ser (P*X*DLS), that is present in all three proteins (13). Aside from this motif, there is very little homology between the N-terminal repression domains of BKLF/KLF3, KLF8, and Ap-2rep/KLF12.

The CtBP co-repressors bind numerous other regulatory proteins, including many conventional DNA-binding proteins, and accessory molecules, such as polycomb and the viral protein E1A (13). The mode of action of CtBP is yet to be fully elucidated. CtBP proteins can recruit histone deacetylases (16, 17), however, deacetylase-independent repression has also been observed. CtBP interacts with polycomb group proteins (18) and proteins such as Ikaros (19) that are contained in chromatin remodeling complexes, suggesting that CtBP proteins may also participate in regulation of gene expression through the non-covalent modification of chromatin structure.

We have found that BKLF/KLF3 is a potent transcriptional repressor, and during our analysis of transcriptional repression by BKLF (14), we noted that abrogation of CtBP recruitment does not entirely abolish this function. This suggested that BKLF may recruit one or more additional cofactors to regulate transcription. A two-hybrid screen against BKLF identified the LIM-only protein FHL3 as another BKLF partner protein.

FHL3 is a member of the recently recognized four and half LIM domain (FHL) family, which consists of FHL1–4, ACT, and KyoT1 (20–26). These proteins are made up of four LIM domains, plus one N-terminal "half" LIM domain whose amino acid sequence resembles that of a single GATA type zinc finger. LIM domains are composed of a double zinc finger motif that co-ordinate two zinc ions and are primarily thought to mediate protein-protein interactions (27). The term LIM originates from the isolation of three *Caenorhabditis elegans* transcription factors, Lin-11, Isl-1, and Mec-3, proteins in which this domain

* This work was supported by a grant from the Australian National Health and Medical Research Council (to M. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 61-2-9351-2233; Fax: 61-2-9351-4726; E-mail: M.Crossley@mmb.usyd.edu.au.

[1] The abbreviations used are: KLF, Sp/Krüppel-like factor; EKLF, erythroid Krüppel-like factor; DBD, DNA binding domain; CtBP, C-terminal-binding protein; FHL, four and half LIM domain; AR, androgen receptor; WT1, Wilms tumor-1; AD, activation domain; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; GSH, glutathione; tk, thymidine kinase; CREB, cAMP-response element-binding protein; CREM, cAMP-responsive element modulator.

was first described (28). FHL1 and 2 are highly expressed in skeletal muscle and have been observed to be present in the cytoplasm. Consequently FHL proteins were originally hypothesized to play a role in cytoskeletal function in muscle cells (24, 25). Recently, however, FHL family members have also been detected in the nucleus, and it now appears that in this compartment several FHL family members function as transcriptional co-regulators. The testis-specific protein ACT serves as a co-activator of CREM and CREB (21), and its restricted expression pattern helps explain how testis-specific gene activation by CREM is mediated. FHL2 has been shown to act as a specific co-activator of the androgen receptor (AR) (23) and the zinc finger protein Wilms tumor-1 (WT1) (29) and as a co-repressor of the multi-zinc finger protein PLZF (30). Sub-cellular partitioning also appears to be important for the regulation of FHL function. FHL2 has been observed in both nuclear and cytoplasmic fractions, and its translocation to the nucleus in response to Rho signaling can lead to potent stimulation of AR-mediated gene expression (31). Additionally, ACT interacts with the kinesin KIF17b, and this interaction is thought to regulate its nuclear/cytoplasmic partitioning (32). To date, the FHL proteins described have been found to behave as conventional transcriptional co-regulators, raising the possibility that they may be involved in recruiting the basal transcriptional machinery or chromatin remodeling enzymes, but their precise mechanism of action has not yet been defined.

Here we show that FHL3 binds BKLF and acts as a co-repressor. We also demonstrate that FHL3 contacts CtBP2, a separate BKLF co-repressor. Because sub-cellular distribution of FHL factors may affect their function, we were interested in examining the cellular location of FHL3. We present evidence that FHL3, CtBP2, and BKLF proteins exist in a large complex only in the nucleus and that FHL3 is significantly enriched in the nucleus only when it is co-expressed with both BKLF and CtBP2. We suggest that FHL3, and other members of the FHL family, operate as linking modules that stabilize multiprotein transcriptional complexes in the nucleus in addition to their reported roles in the cytoplasm.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Assays—The Clontech two-hybrid system was used according to the manufacturer's instructions. A human erythroleukemia (K562) cell cDNA library in the gal4 activation domain (AD) fusion vector, pGAD10, was transfected into the yeast strain HF7c harboring the gal4DBD-BKLF-(1–268) fusion protein expressed from pGBT9. Candidate interactors were re-tested by co-transfecting gal4DBD-bait proteins and gal4AD-prey proteins into HF7c. Transformants were selected on Trp/Leu-deficient plates and patched onto Trp/Leu/His-deficient media. Growth was scored after 30 h. Controls containing bait and prey alone were also conducted and were negative for growth up to more than 72 h.

In Vitro Binding Assays—Full-length *BKLF* and *FHL3* were cloned into the vector pcDNA3 (Invitrogen), and ^{35}S-labeled *in vitro* translated proteins were generated using T7 polymerase and the TnT system from Promega. GST fusion proteins were prepared and binding assays were carried out as previously described (33).

Northern Blotting—A ^{32}P-labeled 300-bp fragment from the coding sequence of *FHL3* was used to probe two commercial filters, a multiple tissue Northern blot containing mRNA from human adult tissues, and a blot containing mRNA from various cancer cell lines, both from Clontech. Hybridization was performed at high stringency, using ExpressHyb (Clontech). These membranes were stripped and re-probed using a human β-actin probe supplied by Clontech.

Mammalian Cell Transfections and Reporter Assays—NIH-3T3 cells were cultured and transfected, using the calcium phosphate method, as described previously, as were chloramphenicol acetyltransferase and growth hormone reporter assays (14). COS cells were cultured and transfected, using the DEAE-dextran method, as previously described (15). K562 stable cell lines expressing *BKLF* were generated as described (34), using the expression vector pEF1α.*neo*.BKLF.

Nuclear and Cytoplasmic Extract Preparation—Nuclear extracts were prepared as previously described (35). Cytoplasmic extracts were prepared during the procedure for preparation of nuclear extracts as above.

Antibody-Bead Cross-linking and Co-immunoprecipitation—To generate antibody-bead conjugates, 100 μl of pre-immune or immune sera was incubated with 100 μl of protein-A-agarose beads (Roche Molecular Biochemicals) to a final volume of 1 ml with PBS, for 60 min at 4 °C. Beads were then washed twice with 10 volumes of 200 mM sodium borate, pH 9.0, and were resuspended in 10 volumes of 200 mM sodium borate, pH 9.0, with dimethylpimilimidate (Sigma) at a final concentration of 20 mM. Cross-linking was performed for 30 min at room temperature, and the reaction was stopped by washing once with 10 volumes of 200 mM ethanolamine, pH 8.0, and then incubation with 200 mM ethanolamine, pH 8.0, for 2 h at room temperature. Beads were then washed twice with 10 volumes of 100 mM glycine, pH 2.8, to remove non-cross-linked antibody, washed once with 10 volumes PBS, and resuspended in PBS.

For immunoprecipitation of nuclear extracts, 80–100 μl of extract from transfected COS cells were diluted 1:3 with Nonidet P-40 buffer (0.5% Nonidet P-40/Igepal, 150 mM NaCl, 50 mM Tris, pH 8.0, 1 μg/μl leupeptin, 1 μg/μl aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The solution was pre-cleared with 10 μl each of protein-A and protein-G beads at 4 °C for 30 min. The supernatant was then immunoprecipitated with 10 μl of anti-BKLF or anti-FHL3 beads, or the corresponding pre-immune beads, with incubation at 4 °C for 1 h. The beads were washed four times with Nonidet P-40 buffer and were resuspended in SDS-PAGE loading buffer with 100 mM dithiothreitol. The samples were treated at 60 °C for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose overnight at 100 mA. Blots were then probed by standard Western blotting techniques with the appropriate primary antibody then bound with the appropriate secondary antibody linked to horseradish peroxidase and were detected using the PerkinElmer Life Sciences Chemiluminescence Reagent *Plus* kit. For immunoprecipitation of cytoplasmic extracts, a higher salt buffer was used during the immunoprecipitation step to maintain the salt concentration equivalent to that used for nuclear extracts. Subsequent washes were performed with the 150 mM NaCl Nonidet P-40 buffer.

Gel-filtration Chromatography—Nuclear and cytoplasmic extracts were applied to a Superose6™ column (Amersham Biosciences) equilibrated with buffer containing 20 mM HEPES, pH 7.9, 10% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA and run at 0.25 ml·min^{-1} at 4 °C. The eluant was monitored at 280 nm. 1-ml fractions were collected, and constituent proteins were precipitated with 0.5 ml of 20% trichloroacetic acid (Sigma). After washing with cold ethanol, samples were resuspended in SDS-PAGE loading buffer, separated by SDS-PAGE, and treated as described above for immunoprecipitation experiments. Protein standards were applied to the column separately (blue dextran, 2000 kDa; thyroglobulin, 670 kDa; ferritin, 440kDa; catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 14 kDa; Amersham Biosciences).

Anti-FHL3 Antibody Generation—A fusion protein between GST and full-length FHL3 was prepared as described (14). The GST fusion protein was then eluted from the GSH-agarose beads using 25 mM glutathione (GSH) in elution buffer (100 mM Tris-HCl (pH 7.5), 120 mM NaCl) for 1 h at 4 °C with rotation. The GSH-agarose beads were pelleted and re-extracted. The eluates were pooled, and the protein concentration was determined. The protein was lyophilized, and four 300-μg doses were used as antigens for rabbit inoculation. Inoculation and sera collection were performed by the Veterinary Services Division of the Institute of Medical and Veterinary Sciences (101 Blacks Rd., Gilles Plains, S.A., 5086, Australia).

RESULTS

BKLF Interacts with the LIM Protein FHL3—We have previously shown that BKLF can recruit the co-repressor protein CtBP2 to repress transcription (14). A short amino acid motif in BKLF, Pro-Val-Asp-Leu-Thr (PVDLT), is required for the interaction between BKLF and CtBP2, and when this motif is disrupted, repression by BKLF is compromised. However, some repression by BKLF is retained even when the PVDLT motif is mutated (14). This suggested that BKLF might recruit additional cofactors to repress transcription. We thus carried out a further two-hybrid screen to identify additional BKLF cofactors.

The bait used in this screen consisted of the gal4DBD fused to amino acids 1–268 of BKLF (encompassing the repression

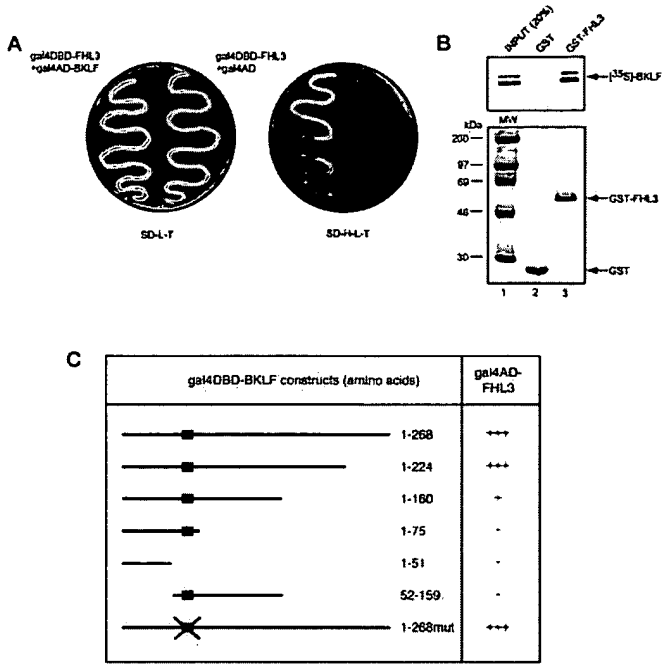

FIG. 1. **BKLF/KLF3 binds the LIM protein FHL3 *in vivo* (in yeast) and *in vitro*.** *A*, yeast two-hybrid assay shows that FHL3 binds BKLF. Yeast carrying the plasmids shown were grown on media lacking leucine and tryptophan (−L −T) (*left panel*) or histidine, leucine, and tryptophan (−H −L −T) (*right panel*). Growth of yeast carrying the gal4DBD-FHL3 and gal4AD-BKLF fusions on -H-L-T medium show that the bait and prey proteins interact. *B*, GST pull-down assay (*upper panel*) shows BKLF and FHL3 interact *in vitro*. ^{35}S-Labeled BKLF was retained by GST-FHL3 protein (*lane 3*), but not by GST alone (*lane 2*). The input (*lane 1*) contains 20% of the radiolabeled BKLF used in the assay. Equivalent amounts of GST proteins, shown in the *lower panel* by Coomassie Blue staining, were used in each case. *Lane 1* in the *lower panel* contains molecular weight markers (MW). *C*, mapping of the domain in BKLF that binds FHL3. Yeast were co-transformed with the gal4AD-FHL3 plasmid and the gal4DBD fusions with the BKLF deletion constructs shown. The *black box* represents the PVDLT motif in BKLF, and the 1–268mut construct carries a mutation in this motif that abolishes CtBP binding. Interactions, as measured by growth of yeast carrying the plasmids on SD-H-L-T medium, are shown in the *right column*.

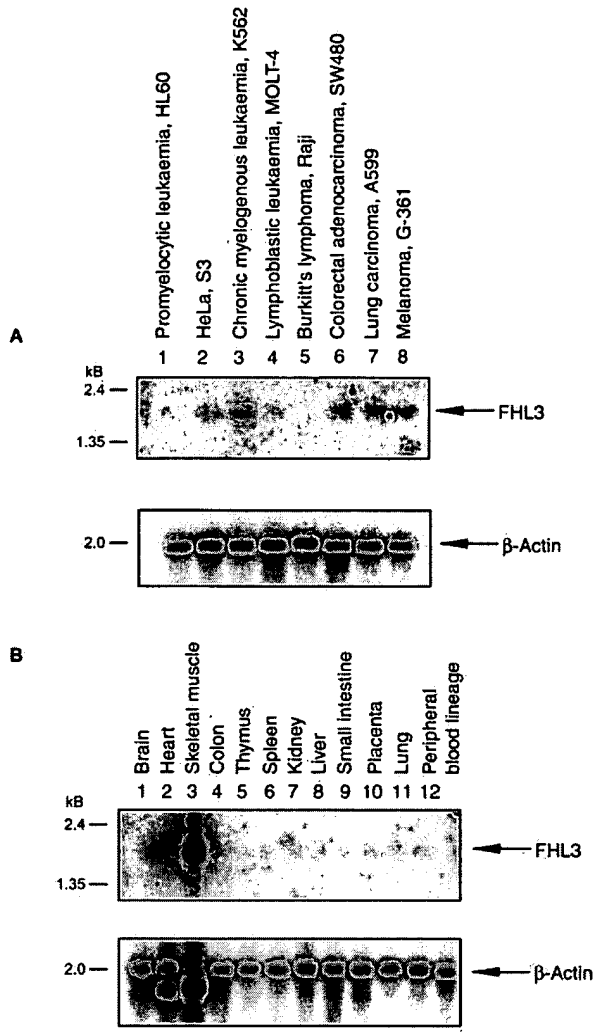

FIG. 2. ***FHL3* mRNA is expressed in K562 (erythroleukemia) cells and is highly expressed in adult heart tissue.** *A*, *FHL3* message is expressed in K562 cells and other cancer cell lines. A Northern blot carrying mRNA from various cancer cell lines (Clontech) was probed with a radiolabeled probe from the coding sequence of *FHL3*. *B*, *FHL3* message is expressed in adult skeletal muscle. A Northern blot carrying mRNA from various adult human tissues (Clontech) was probed with a radiolabeled probe from the coding sequence of *FHL3*. For *A* and *B*, both membranes were stripped and re-probed with a human β-actin probe (Clontech) (*lower panels*) to test for equal loading of mRNA on the membrane.

domain of BKLF (14)). We screened a human erythroleukemia cell (K562) cDNA library. K562 cells express embryonic and fetal globins and are frequently used as a model of erythroid cells at the fetal stage of development (36, 37). This library was used as BKLF is highly expressed in erythroid cells (35) and is believed to play a role in hematopoiesis (38, 39). We screened 4×10^5 clones: 132 primary *His*$^+$ colonies were isolated, 10 of which were also positive for the *lacZ* reporter. Sequencing revealed two clones that carried an 899-bp cDNA insert that matched the human gene termed four and a half LIM protein 3 (*FHL3*) (25). As a first test for the specificity of the interaction, these isolates were tested against the gal4DBD alone and negative control baits,' including gal4DBDs fused to p53, the N finger of GATA-1, and lamin C protein. No interactions were observed (data not shown). We also swapped FHL3 into the bait plasmid and tested the ability of a gal4DBD-FHL3 bait to interact with a gal4AD-BKLF prey. As shown in Fig. 1*A* the interaction is again observed, whereas there is no interaction with the negative control prey, gal4AD alone.

The clones we isolated lacked half of the C-terminal LIM domain of FHL3. To isolate the full-length clone, primers were designed to the 5′- and 3′-ends of the coding sequence (GenBank™ accession number HSU60116), and the K562 cDNA library was used as a template in a polymerase chain reaction. The resulting product was cloned, and sequencing confirmed the presence of a full-length cDNA encoding human FHL3.

To verify this interaction in another system, a GST-FHL3 fusion protein was produced by expression in *Escherichia coli*. The protein was immobilized on GSH-agarose beads (Fig. 1*B*, *lower panel*) and mixed with radiolabeled BKLF generated by *in vitro* transcription and translation in the presence of [^{35}S]methionine. GST-FHL3 strongly retained [^{35}S]BKLF (Fig. 1*B*, *lane 3*, *upper panel*), whereas a negative control protein GST alone retained no BKLF protein (*lane 2*, *upper panel*). Thus, BKLF can interact with FHL3 in both a yeast two-hybrid assay, as either bait or prey, and in GST pull-down assays.

FHL3 mRNA Expression in Cell Lines and Adult Tissue—Because *FHL3* was first identified as a gene that was highly expressed in muscle cells, we wished to test whether it was expressed at appreciable levels in K562 and other selected cells. We probed a Northern blot containing RNA from various cancer cell lines, including K562 cells, with a probe generated from a portion of the coding sequence of *FHL3* (Fig. 2*A*). *FHL3* message was detected in the K562 sample, and low level expression was also found in HeLa (fibroblast), SW480 (colorectal

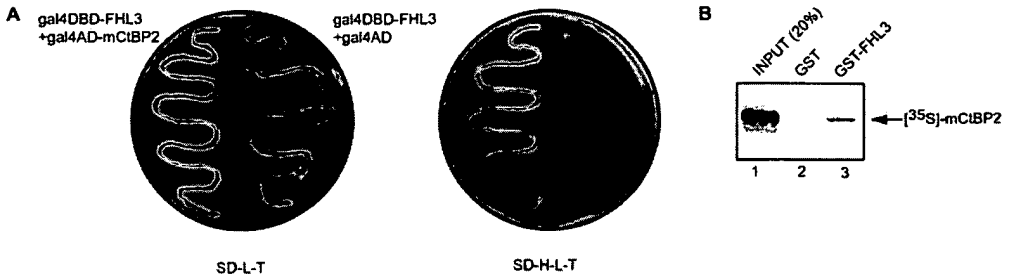

FIG. 3. **FHL3 binds the co-repressor CtBP2 *in vivo* (in yeast) and *in vitro*.** *A*, Yeast two-hybrid assay shows that FHL3 binds CtBP2. Yeast carrying the plasmids shown were grown on medium lacking leucine and tryptophan (*left panel*) or histidine, leucine, and tryptophan (*right panel*). Growth of yeast carrying the gal4DBD-FHL3 and gal4AD-CtBP2 fusions on SD-H-L-T medium show that the bait and prey proteins interact. *B*, GST pull-down assay shows FHL3 and CtBP2 interact *in vitro*. ^{35}S-Labeled CtBP2 was retained by GST-FHL3 protein (*lane 3*) but not by GST alone (*lane 2*). The input (*lane 1*) contains 20% of the radiolabeled CtBP2 used in the assay. GST proteins used were the same as shown in Fig. 1*B*, *lower panel*.

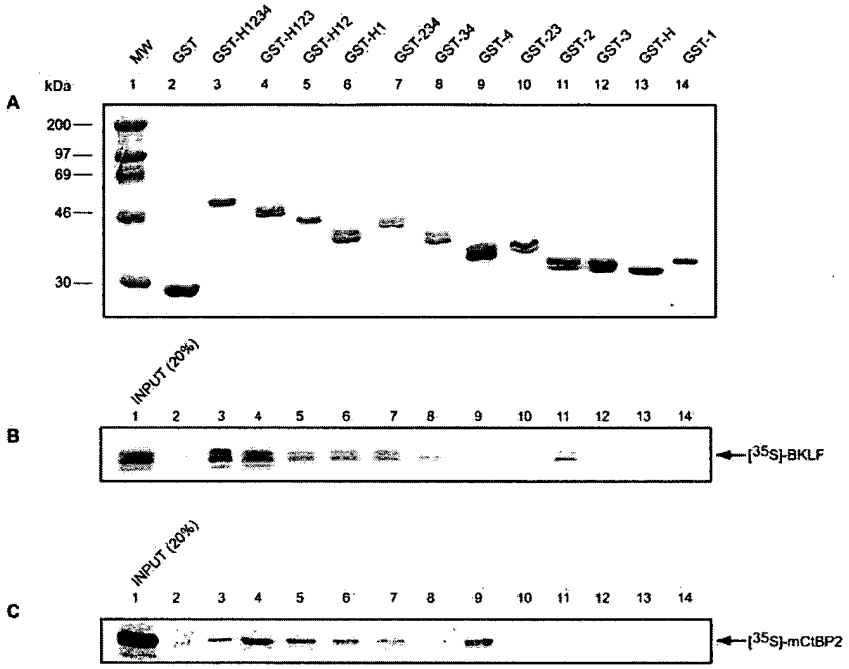

FIG. 4. **Mapping of the domains in FHL3 that bind BKLF and CtBP2.** *A*, GST fusion proteins were prepared with various combinations of LIM domains from FHL3 and tested for binding to ^{35}S-labeled BKLF (*B*) and ^{35}S-labeled CtBP2 (*C*) using GST pull-down assays. Results are summarized in Table I. *Lane 1* in *A* contains molecular weight markers, and the *Input lanes* (*lane 1*) in *B* and *C* contain 20% of the radiolabeled BKLF and CtBP2, respectively, used in the assay. Equivalent amounts of GST-fusion proteins were used in each assay.

adenocarcinoma), A599 (lung carcinoma), and MOLT-4 (melanoma) RNA. We also probed a Northern blot of mRNA from various adult tissues and observed high level expression in skeletal muscle as well as lower levels in heart (Fig. 2*B*). Thus, *FHL3* mRNA is present not only in muscle tissue, as previously observed, but in erythroid cells of fetal type and other cancer cell lines. This result is consistent with other reports that FHL proteins function in several different cell types in addition to muscle cells.

Mapping the Interacting Domains of BKLF and FHL3—BKLF interacts with CtBP2 through a PVDLT motif centered around amino acid 72 (14). To determine whether FHL3 interacts with BKLF through this CtBP-contact region or through a distinct region, a series of BKLF deletions was generated. These constructs produce gal4DBD-BKLF fusion proteins and were tested for their ability to interact with gal4AD-FHL3 in the yeast two-hybrid system (Fig. 1*C*). This experiment indicated that FHL3 interacts strongly with BKLF in a region that lies between amino acids 160 and 224, but that amino acids 75–160 also contribute to binding. Importantly, this region is distinct from the domain in BKLF that binds CtBP2 (amino acids 51–75 (14)), and mutation of the PVDLT motif, which abrogates CtBP2 binding, has no effect on the interaction of BKLF with FHL3 (Fig. 1*C*). This raises the possibility that BKLF may be able to interact with both FHL3 and CtBP2 simultaneously, and the proximity of the FHL3 and CtBP2 binding sites within BKLF suggested that FHL3 might also make contacts with CtBP2 (see below).

FHL3 Also Interacts with CtBP2—To determine whether FHL3 could bind CtBP2, we first used the yeast two-hybrid assay. We tested whether a gal4DBD-FHL3 protein could interact with a gal4AD-CtBP2 protein. A strong interaction was observed between FHL3 and CtBP2, and no interaction was seen with the gal4AD alone (Fig. 3*A*). This interaction was also observed in a GST pull-down assay (Fig. 3*B*), as GST-FHL3 retained radiolabeled CtBP2 (*lane 3*), whereas GST alone did not (*lane 2*). The retention of radiolabeled CtBP2 by FHL3 in GST pull-down assays was generally low but was reproducible (see also Fig. 4*C*). Thus, FHL3 can also interact with CtBP2 in both a yeast two-hybrid assay and in GST pull-down assays. Interestingly, although the vast majority of CtBP-interacting proteins contain a PXDLS-like amino acid motif required for binding CtBP (13), FHL3 has no obvious PXDLS motif (see "Discussion").

Mapping the Interaction between FHL3 and BKLF and CtBP2—We used both GST pull-down assays and the yeast two-hybrid assay to determine which domains in FHL3 interacted with BKLF and which with CtBP2. A series of GST-FHL3 deletion constructs were generated and used in GST pull-down assays with radiolabeled BKLF and CtBP2 (Table I, and Fig. 4,

TABLE I

Mapping of the LIM domains in FHL3 that bind BKLF and CtBP2 and that homodimerize with full-length FHL3, using GST pull-down assays and yeast two-hybrid assays

GST pull-down data are summarized from Fig. 4. Yeast were co-transformed with the gal4DBD-FHL3 constructs as shown, together with the gal4AD fusions with either BKLF-(1–268) or CtBP2 fusions. Interactions, as measured by growth of yeast carrying the plasmids on SD-H-L-T media, are shown in the *right columns*.

FHL3 constructs	Interaction with BKLF: Yeast	Interaction with BKLF: GST pulldown	Interaction with mCtBP2: Yeast	Interaction with mCtBP2: GST pulldown	Interaction with FHL3: Yeast
H1234	+++	+++	+++	+	+++
H123	+++	++	+++	++	+
H12	+	+	++	+	+
H1	-	+	+	+	-
234	-	+	-	-	-
34	++	-	++	-	++
4	-	-	-	+	-
23	-	-	-	-	-
2	-	+	-	-	-
3	-	-	-	-	-
H	-	-	-	-	-
1	-	-	-	-	-

B and *C*). The same domains of FHL3 were also used in the yeast two-hybrid system, as gal4DBD fusions, and were tested against galAD fusions with both BKLF (amino acids 1–268) and CtBP2. A summary of the GST pull-down assays and yeast two-hybrid assays is shown in Table I. The results indicate that LIM domains half, 1, and 2 (H–1–2) are sufficient for interaction with BKLF, whereas, only LIM domains half and 1 of FHL3 are sufficient for the interaction with CtBP2. Other domains of FHL3, however, also appear to contact BKLF and CtBP2 (for instance LIM domains 3–4 contact both BKLF and CtBP2, and LIM domain 4 contacts CtBP2), and in some instances results of the yeast two-hybrid assay and the GST pull-down assays are not consistent. Overall, these experiments suggest that folding of the entire FHL3 protein may be important and that the complete binding surfaces may be more complex than just one discreet contiguous domain. Our results are similar to previous attempts to map the interaction between FHL2 and the AR (23) or PLZF (30). In these cases, deletion studies showed that the LIM domains H–1–2 and 3–4 of FHL2 both interact with the AR with similar affinities (22), and no single LIM domain of FHL2 was capable of interacting with PLZF (29). Taken together these experiments are consistent with the view that the four and a half LIM domains in these proteins fold into a complex protein interaction surface that may wrap around and make multiple specific contacts with its partner proteins.

FHL3 Can Homodimerize—It has previously been reported that FHL proteins can dimerize (22). We tested whether this was the case for FHL3. A strong interaction was observed between the proteins gal4DBD-FHL3 and gal4AD-FHL3 in the yeast two-hybrid assay. Deletion studies show that LIM domains H–1–2 of FHL3 are sufficient for contact with full-length FHL3 but that dimerization can also occur between LIM domains 3–4 and full-length FHL3 (Table I). Again this result demonstrates that the series of contact surfaces is likely to be complex. A full understanding of the contact surfaces will require structural analysis of the protein complexes.

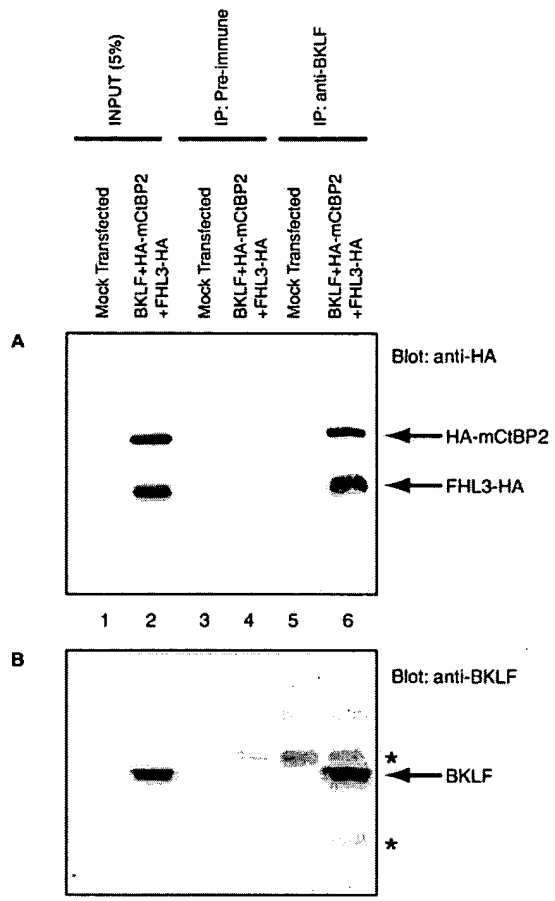

FIG. 5. **BKLF binds both FHL3 and CtBP2 *in vivo*.** COS cells were co-transfected with expression vectors for BKLF, and HA-tagged CtBP2 and FHL3, or were mock transfected. Nuclear extracts were immunoprecipitated with either anti-BKLF antibody (*lanes 5* and *6*) or pre-immune Ig (*lanes 3* and *4*), and the Western blot was probed with anti-HA antibody (*upper panel*), stripped and re-probed with anti-BKLF antibody (*lower panel*). Five percent of the extract used for the immunoprecipitations is shown in *lanes 1* and *2*. The *asterisk* shows the immunoglobulin heavy and light chains.

BKLF, CtBP2, and FHL3 Form a Nuclear-specific Complex in Vivo—We next used co-immunoprecipitation assays to assess the *in vivo* interactions between BKLF, CtBP2, and FHL3 (Fig. 5). COS cells were co-transfected with pMT2 expression vectors for BKLF, plus HA-tagged CtBP2 and HA-tagged FHL3, or were mock transfected with equivalent amounts of empty vector. Nuclear extracts were prepared 48 h post-transfection and were immunoprecipitated with either anti-BKLF antibody or pre-immune anti-sera. Immunoprecipitates were probed by Western blotting with anti-HA antibody. Both CtBP2 and FHL3 are efficiently immunoprecipitated by anti-BKLF antibody (Fig. 5*A*, *lane 6*), whereas neither protein is immunoprecipitated with pre-immune sera (*lane 4*). Additionally, CtBP2 and FHL3 are not precipitated from mock transfected cells with either anti-BKLF or pre-immune sera (*lanes 3* and *5*). This same membrane was subsequently stripped and re-probed with anti-BKLF antibody (Fig. 5*B*). As expected, BKLF is efficiently immunoprecipitated with anti-BKLF antibody (Fig. 5*B*, *lane 6*) but not with pre-immune sera (*lane 4*), and no BKLF protein was precipitated from mock transfected cells (*lanes 3* and *5*). These results are consistent with previous *in vitro* experiments and confirm that BKLF can associate with both CtBP2 and FHL3 *in vivo*.

To further understand the interaction between BKLF, CtBP2, and FHL3, we used native gel-filtration chromatography to analyze whether these three proteins co-elute (Fig. 6). In

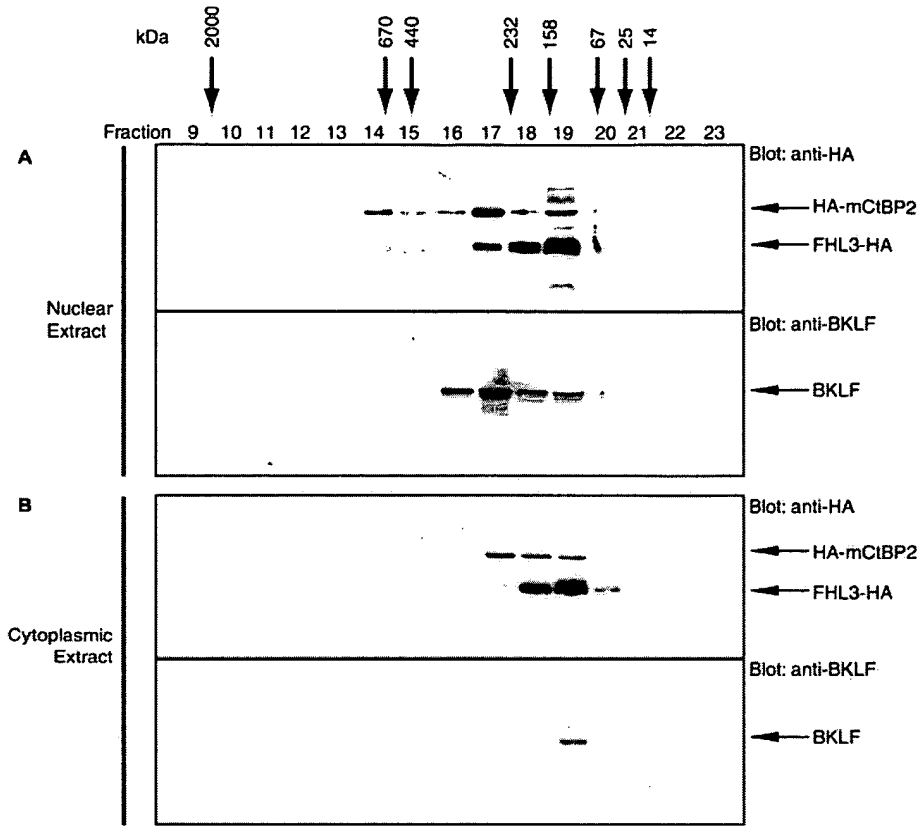

FIG. 6. **BKLF, FHL3, and CtBP2 are part of a nuclear-specific complex of ~240 kDa.** COS cells were co-transfected with expression vectors for BKLF and HA-tagged CtBP2 and FHL3. Nuclear (*A*) and cytoplasmic (*B*) extracts were prepared and separated by native gel-filtration chromatography. Proteins in each fraction were precipitated, and probed by Western blotting, first with anti-HA antibody (*upper panel* in *A* and *B*), then stripped and re-probed with anti-BKLF antibody (*lower panel* in *A* and *B*). Nuclear BKLF, FHL3, and CtBP2 first co-elute in fraction 17, at greater than 232 kDa, whereas the elution profile for cytoplasmic BKLF, FHL3, and CtBP2 is distinctly different, with the three proteins predominantly co-eluting in fraction 19, between 67 and 158 kDa.

this experiment we analyzed first nuclear and then equivalent amounts of cytoplasmic extract. Extracts were separated by gel-filtration chromatography, and the fractions were precipitated with trichloroacetic acid, separated by SDS-PAGE, and probed by Western blotting with anti-HA antibody (to detect HA-CtBP2 and FHL3-HA), then stripped and re-probed with anti-BKLF antibody. The monomer masses of BKLF, CtBP2, and FHL3 are 39, 49, and 31 kDa, respectively, but as described above FHL3 can self-associate, as can CtBP2 (14) and BKLF.[2] Analysis of nuclear extracts indicated that BKLF, CtBP2, and FHL3 first co-elute in fraction 17, with BKLF and CtBP2 peaking in this fraction (Fig. 6*A*). Examination of standards included in the experiment suggest that fraction 17 contains protein in excess of 232 kDa, so the most simple explanation is that this complex may consist of dimers of each protein (expected molecular mass, 239 kDa). All three proteins also co-elute in fraction 19, between the 67- and 158-kDa standards, with significant amounts of FHL3 found in this fraction. This fraction may contain all three proteins eluting independently as homodimers. Interestingly, CtBP2 and FHL3 also co-elute independently of BKLF in fraction 14 and 15 between the 440- and 670-kDa standards, suggesting these two proteins may be found in additional higher molecular weight complexes within the nucleus. The elution profile from cytoplasmic extracts is significantly different (Fig. 6*B*). In particular, the majority of BKLF elutes in fraction 19 (*i.e.* between 67 and 158 kDa) and FHL3 is predominantly found in fractions 18 and 19. This observation suggests that a complex of around 240 kDa containing BKLF, CtBP2, and FHL3 may form in the nucleus but not in the cytoplasm.

To confirm this, co-immunoprecipitations were performed with cytoplasmic extract from COS cells transfected with BKLF, HA-CtBP2, and FHL3-HA using anti-BKLF antibodies,

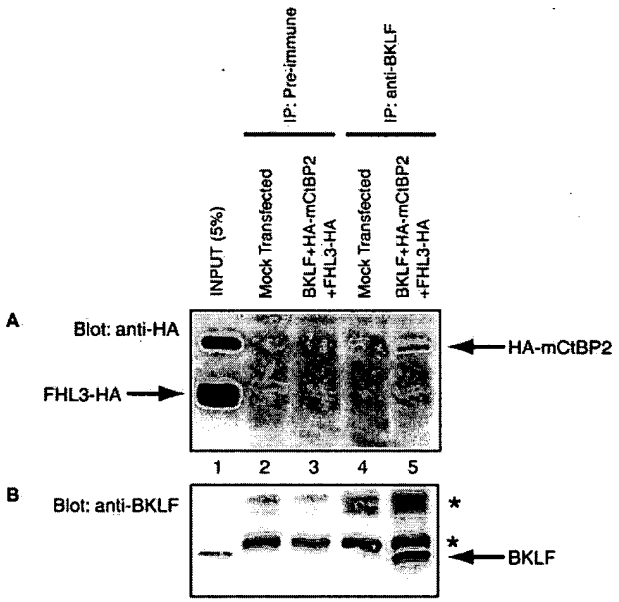

FIG. 7. **BKLF does not bind FHL3 in the cytoplasm.** COS cells were co-transfected with expression vectors for BKLF, and HA-tagged CtBP2 and FHL3, or were mock transfected. Cytoplasmic extracts were immunoprecipitated with either anti-BKLF antibody (*lanes 4* and *5*) or pre-immune Ig (*lanes 2* and *3*), and the Western blot was probed with anti-HA antibody (*upper panel*), stripped, and re-probed with anti-BKLF antibody (*lower panel*). *Lane 1* contains 5% of the extract used for the immunoprecipitation. The *asterisk* shows the immunoglobulin heavy chain.

or pre-immune anti-sera (Fig. 7). Immunoprecipitates were probed with anti-HA antibody (Fig. 7*A*). No FHL3 protein is found to be associated with BKLF in the cytoplasmic extracts (Fig. 7*A*, *lane 5*), and only very small amounts of CtBP2 (*lane 5*). Additionally, neither CtBP2 nor FHL3 is immunoprecipi-

[2] J. Turner, H. Nicholas, D. Bishop, J. M. Matthews, and M. Crossley, unpublished results.

tated with pre-immune sera (*lane 3*), or from mock transfected cells (*lanes 2* and *4*). The blot was stripped and re-probed with anti-BKLF antibody, showing that BKLF is efficiently precipitated from cytoplasmic extract (Fig. 7*B*, *lane 5*). Thus, the *in vivo* interaction between BKLF and both FHL3 and CtBP2 seen in the nucleus (above) is not observed in the cytoplasm.

Co-expression of BKLF and CtBP2 Leads to the Nuclear Enrichment of FHL3—CtBP proteins are found distributed throughout the cell and have been hypothesized to have roles both in the nucleus and the cytoplasm (13). As shown above the DNA-binding protein BKLF is also detectable in both the nucleus and the cytoplasm, although it is more abundant in the nucleus (Fig. 6). Previous work on FHL proteins has also suggested that they exist in both the nucleus and the cytoplasm (20–23, 31, 40–42). Moreover, it has been shown that Rho-signaling can promote nuclear enrichment of FHL2 protein (31). Thus, it appears that the sub-cellular localization of FHL proteins may be subject to regulation. In the gel-filtration experiments discussed above, we noted that the elution profile for FHL3 is markedly different between nuclear and cytoplasmic fractions and that BKLF, CtBP2, and FHL3 only co-elute in high molecular weight fractions in nuclear extracts. We thus examined the levels of BKLF, CtBP2, and FHL3 in the nucleus and cytoplasm when different combinations of these three proteins are co-expressed in COS cells.

When FHL3 is expressed on its own, it is found predominantly in the cytoplasm (Fig. 8*A*, compare *lanes 1* and *2*). When co-expressed with CtBP2, again, the majority of FHL3 is found in the cytoplasm, as is CtBP2 (compare *lanes 3* and *4*). When FHL3 is co-expressed with BKLF, an increased fraction of FHL3 is found in the nucleus, although it is still primarily cytoplasmic, whereas BKLF is predominantly nuclear (Fig. 8*A*, *upper* and *lower panels*, compare *lanes 5* and *6*). However, when FHL3 is co-expressed with both BKLF and CtBP2, FHL3 is significantly enriched in the nucleus, as is CtBP2 (Fig. 8*A*, *upper* and *lower panels*, compare *lane 7* with *1* and *3*). Taken together with the co-immunoprecipitation assays and gel-filtration data above, it appears that a specific complex of ~240 kDa containing BKLF, CtBP2, and FHL3 may accumulate in the nucleus only when all three proteins are present and that this complex is not present in the cytoplasm.

To examine whether BKLF promotes nuclear accumulation of endogenous FHL3 and CtBP2 protein, we generated four stable cell lines expressing BKLF using K562 hematopoietic cells, which normally express very low levels of BKLF protein (Fig. 8*B*, *lane 1*, *upper panel*). K562 cells express *FHL3* mRNA message (see above), and the original FHL3 prey plasmid, and full-length *FHL3* were isolated from a K562 cell cDNA library. We generated an anti-FHL3 antibody and used this to test the effect of BKLF expression on nuclear localization of FHL3. The four K562 cell lines carrying the stable transgene for *BKLF* show high level BKLF expression in the nucleus, compared with the parental line (Fig. 8*B*, *upper panel*, compare *lane 1* with *lanes 2–5*). Importantly, these same BKLF stable cell lines show a marked increase in FHL3 protein in nuclear extracts, compared with the parental cell line (Fig. 8*B*, *lanes 1–5*, *middle panel*). Additionally, nuclear levels of CtBP2 are increased in the BKLF stable lines (Fig. 8*B*, *lanes 1–5*, *lower panel*). Thus, in a similar manner to that observed in transfected COS cells, BKLF can promote the nuclear accumulation of endogenous FHL3 and CtBP2 in erythroleukemia cells.

FHL3 Can Repress Transcription—Previous studies have shown that FHL proteins can function as transcriptional regulators. The FHL protein ACT serves as co-activator of CREM and CREB (21), and FHL2 as a co-regulator for the androgen receptor (22, 23), WT1 (29), and PLZF (30). We thus examined

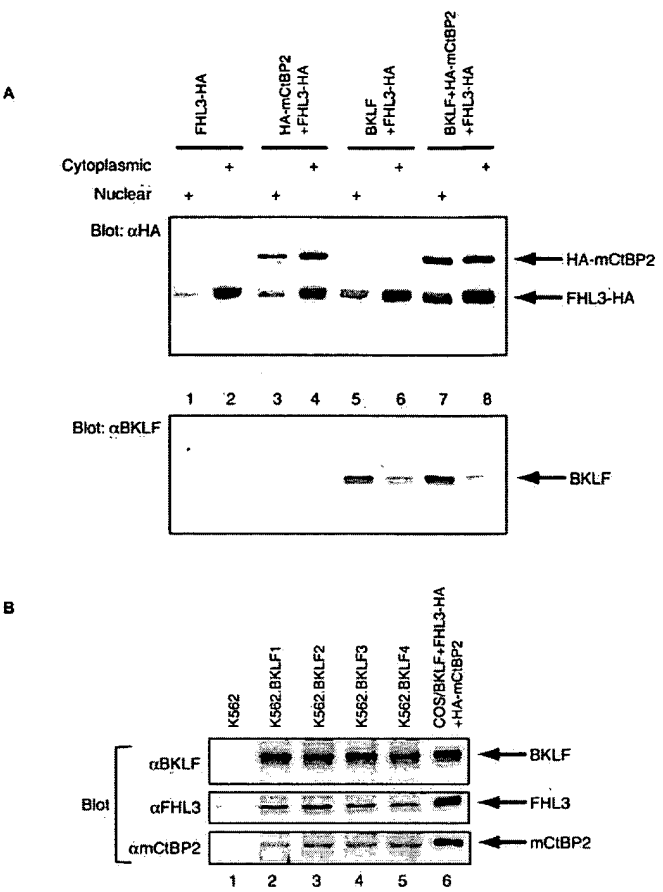

FIG. 8. **Co-expression of BKLF, FHL3, and CtBP2 promotes nuclear localization of FHL3 and CtBP2.** *A*, COS cells were transfected with expression vectors as shown, and nuclear and cytoplasmic extracts were prepared and probed by Western blotting as shown. FHL3 is predominantly cytoplasmic (*upper panel*) when expressed on its own (*lanes 1* and *2*) or co-expressed with either CtBP2 (*lanes 3* and *4*) or BKLF (*lanes 5* and *6*). When FHL3 is co-expressed with both BKLF and CtBP2, FHL3 is significantly enriched in the nucleus, as is CtBP2 (*lanes 7* and *8*, *upper panel*) and BKLF (*lanes 7* and *8*, *lower panel*), albeit to a lesser extent. *B*, stable expression of BKLF in K562 cells promotes nuclear accumulation of endogenous FHL3 and CtBP2. Nuclear extracts were prepared from four K562 cell lines carrying stable BKLF transgenes and parental K562 cells and were probed by Western blotting as shown. BKLF stable lines (*lanes 2–5*, *upper panel*) express high levels of BKLF protein in the nucleus, whereas K562 cells express little BKLF (*lane 1*, *upper panel*). FHL3 expression in the nucleus is significantly increased in the nucleus in the BKLF stable cell lines (*lanes 1–5*, *middle panel*), as is CtBP2 (*lanes 1–5*, *lower panel*). *Lane 6* contains nuclear extract form COS cells transfected with expression vectors for BKLF, FHL3-HA, and HA-mCtBP2 (note that the small discrepancy in migration between HA-mCtBP2 and endogenous CtBP2 is due to addition of the HA tag).

the transcriptional activity of FHL3 (Fig. 9). Because FHL proteins are not known to bind DNA, we first fused FHL3 to the gal4DBD and tested its ability to regulate basal transcription of two artificial test promoters carrying gal4 binding sites. The gal4DBD-FHL3 fusion protein repressed the thymidine kinase (tk) promoter by greater than 12-fold in a dose-dependent manner (Fig. 9*A*, *columns 5–7*), whereas the gal4DBD alone has no effect on this promoter (Fig. 9*A*, *columns 2–4*). Additionally, the FHL3 fusion protein repressed the minimal promoter containing only a TATA box to about 15-fold (Fig. 9*B*, *columns 5–7*), again in a dose-dependent manner, whereas the gal4DBD only marginally affected its activity (Fig. 9*A*, *columns 2–4*).

We next tested the ability of FHL3 to regulate activated transcription in the context of a natural promoter. The erythropoietin promoter is strongly activated by the erythroid-specific transcription factor GATA-1 (43). The promoter contains

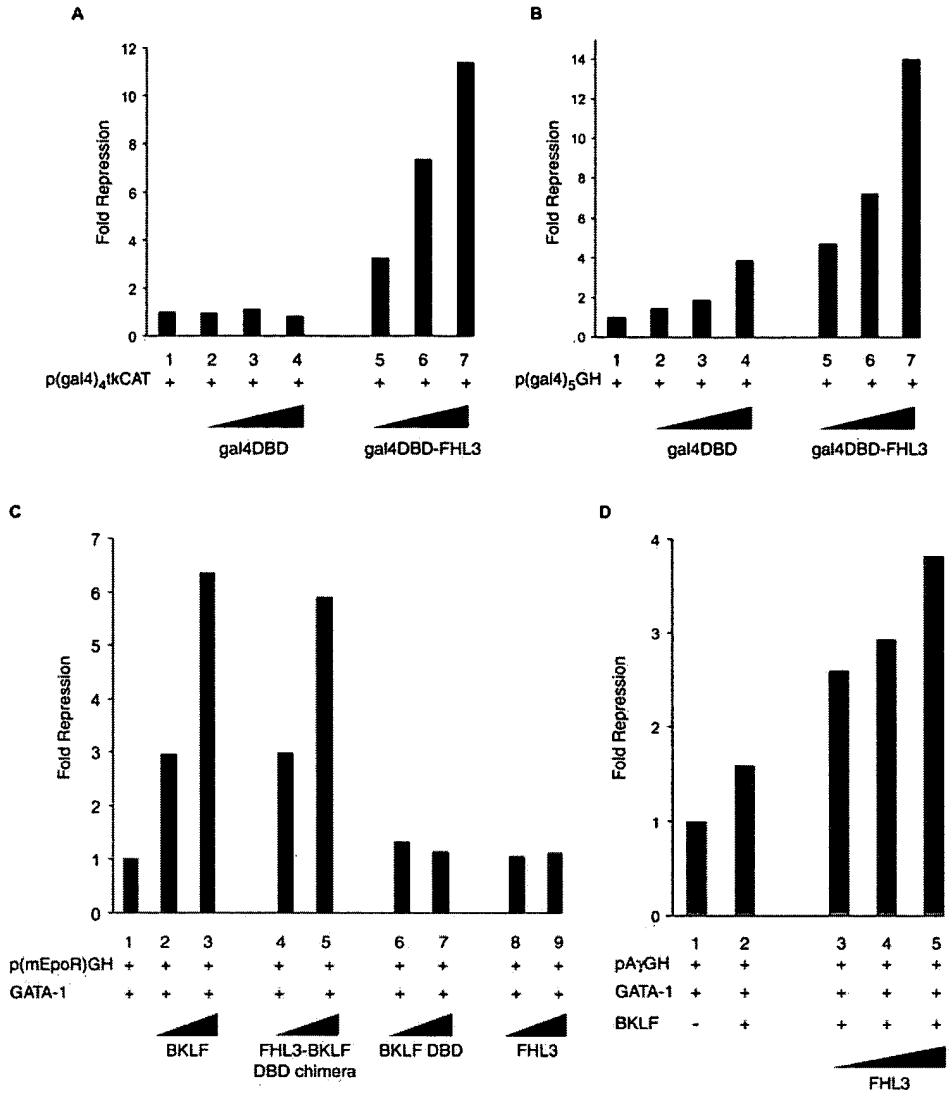

FIG. 9. **FHL3 can repress both basal and activated transcription when tethered to a promoter with a heterologous DNA binding domain, and can potentiate BKLF repression.** *A*, FHL3 represses the thymidine kinase (tk) promoter. NIH-3T3 cells were transfected with 5 μg of the reporter plasmid p(gal4)$_4$tkCAT (*column 1*), along with 0.5, 2, and 4 μg of expression vectors for the gal4DBD (*columns 2–4*) or a fusion between the gal4DBD and FHL3 (*columns 5–7*). The gal4DBD-FHL3 fusion efficiently repressed the basal activity of this promoter, whereas the gal4DBD alone has no effect. *B*, FHL3 represses a basal promoter carrying the β-globin TATA box. NIH-3T3 cells were transfected with 5 μg of the reporter plasmid p(gal4)$_5$GH (*column 1*) along with 0.5, 2, and 4 μg of expression vectors for the gal4DBD (*columns 2–4*) or a fusion between the gal4DBD and FHL3 (*columns 5–7*). The gal4DBD-FHL3 fusion efficiently repressed the basal activity of this promoter, whereas the gal4DBD alone has minimal effect on the promoter. *C*, FHL3 represses the erythropoietin promoter, when activated by GATA-1. NIH-3T3 cells were transfected with 1 μg of the reporter plasmid p(EpoR)GH and 1 μg of the GATA-1 expression vector pRcCMV.GF1 (*columns 1–9*) together with 0.5 and 1 μg of expression vectors for BKLF (*columns 2* and *3*), the FHL3-BKLF DBD chimera (*columns 4* and *5*), the DBD of BKLF (*columns 6* and *7*) or FHL3 (*columns 8* and *9*). *D*, FHL3 potentiates repression by BKLF. COS cells were transfected with 200 ng of the reporter plasmid pAγGH and 2 μg of the GATA-1 expression vector pXM.GF1 (*columns 1–5*) together with 100 ng of the expression vector pcDNA3.BKLF (*columns 2–5*) and the 500 ng and 1 and 2 μg of the FHL3 expression vector pMT2.FHL3-HA (*columns 3–5*).

multiple CACCC elements and we have found that BKLF can potently repress GATA-1-mediated activation (Fig. 9*C*, *columns 1–3*).[2] The level of GATA-1 activation of the basal activity of this promoter is ~20-fold (Fig. 9*C*, *column 1*) and is assigned a -fold repression of 1. BKLF represses the promoter ~7-fold (Fig. 9*C*, *columns 2–3*). We tested the ability of FHL3 to repress GATA-1-mediated activation of this promoter. To direct FHL3 to the promoter, we generated a chimeric fusion protein between FHL3 and the zinc finger DBD of BKLF. As judged by electrophoretic mobility shift assays, this FHL3-zinc finger fusion protein can efficiently bind CACCC sites in DNA in a similar manner to BKLF (data not shown). As shown in Fig. 9*C* (*columns 4* and *5*), the FHL3-zinc finger chimera represses GATA-1 activation as efficiently as BKLF, whereas FHL3 alone (*columns 8* and *9*) and the BKLF zinc finger DBD alone (*columns 6* and *7*) have no effect on GATA-1 activation.

To further test the activity of FHL3, we examined whether FHL3 (without a linked DBD) could potentiate BKLF repression (Fig. 9*D*). GATA-1 strongly activates the erythroid-specific Aγ-globin promoter, a known GATA-1 target gene (44). Again, GATA-1 activation of this promoter, at ~20-fold, is set to a -fold repression of 1 (Fig. 9*D*, *column 1*). We have found that BKLF can potently repress this natural promoter, through binding to multiple CACCC sites in the promoter.[2] As shown in Fig. 9*D* (*column 2*), low levels of BKLF expression vector (100 ng) repress GATA-1 activity ~1.5-fold. Note that a low level of BKLF expression was chosen for minimal repression of the promoter, allowing further super-repression by FHL3 to be analyzed. Importantly, when FHL3 is co-expressed with BKLF (Fig. 9*D*, *columns 3–5*), BKLF repression is enhanced up to

4-fold. Thus FHL3 can repress both basal and activated transcription when targeted to a promoter through heterologous DNA binding domains, and the native FHL3 protein can potentiate BKLF repression. This fits well with recent data, showing that FHL3 (and FHL2) can also act as co-repressors for the multi-zinc finger repressor PLZF (30).

DISCUSSION

Transcriptional regulation is mediated in part by the recruitment of multiprotein complexes to regulatory regions in DNA. Revealing the composition and activities of these complexes will aid our understanding of how DNA-binding transcription factors regulate gene expression in a tissue- and temporal-specific manner. Here we show that the Krüppel-like factor BKLF/KLF3 can bind to two co-regulatory molecules, FHL3 and CtBP2, both of which can repress transcription. This work expands our understanding of the activities of the FHL class of proteins: FHL proteins were previously known to behave primarily as co-activators; we show here that FHL3 can behave as a transcriptional repressor and forms a physical link between BKLF and its co-repressor CtBP2.

FHL proteins were initially identified as being highly expressed in muscle cells (24, 25) and were originally proposed to play roles in cytoskeletal regulation. This proposal was primarily based on the observation that, like the cytoskeletal-associated LIM proteins zyxin and paxillin, the FHL proteins were also composed primarily of LIM domains, and were developmentally regulated during muscle hypertrophy (24). Recently, however, it has become clear that many FHL proteins participate in the regulation of gene expression within the nucleus where they serve as co-regulators of DNA-binding transcription factors. ACT binds the testis-specific factor CREM (21), and ACT, FHL2, and FHL3 all bind CREB (22). Additionally, FHL2 binds Wilms Tumor-1 (WT-1) (29), and the androgen receptor (in an agonist-dependent manner) (23). In these cases, the FHL proteins behaved as co-activators, but whether they act as conventional co-activators, recruiting components of the basal transcriptional machinery, or chromatin-modifying proteins or act in a different manner has not been established. There are also two reports of FHL proteins functioning to counter gene activation. KyoT2, a splice variant of FHL1, antagonizes the activity of RBP-J by displacing co-activators EBNA2 or Notch (20). Furthermore, FHL2 has been demonstrated to function as a co-repressor of the multi-zinc finger repressor PLZF (30), and in this same report, FHL3 was also shown to function as a PLZF co-repressor. These data support our observations that FHL3 binds the transcriptional repressor BKLF and its co-repressor CtBP2 and aid in the direct repression of transcription by BKLF. The finding that FHL proteins participate both in activation and repression fits best with the view that FHL proteins are neither primarily co-activators or co-repressors but, rather, serve as adaptor molecules that stabilize large transcriptional complexes with differing activities.

We have previously shown that BKLF recruits the protein CtBP2 as a co-repressor and that this interaction is dependent on the integrity of a PXDLS-CtBP recognition element in the repression domain of BKLF (14). CtBP proteins have been shown to function as co-repressors for a large number of regulatory molecules that contain PXDLS motifs in their repression domains (13). Having observed that CtBP2 binds FHL3, we searched the FHL3 sequence for PXDLS type sequences that might mediate the interaction. None were detected. This suggests that FHL3 binds a distinct region of CtBP proteins rather than occupying the putative PXDLS peptide pocket of CtBP (13, 45). Deletion analysis of FHL3 is consistent with this view, because no single small peptide in FHL3 contacts CtBP2. Instead, it appears that several different LIM domains of FHL3 are required for contact, and it is likely that different LIM domains of FHL3 may make several contacts with different surfaces of CtBP2. BKLF binds CtBP2 using a PVDLT motif (amino acid residues 70–75) (14) and binds FHL3 through a domain that may extend from around amino acids 80–260. FHL3 also uses several different LIM domains to contact BKLF. The mode of contact utilized by FHL3 is reminiscent of that seen with other members of the FHL family that also seem to utilize multiple different LIM domains to contact their partner proteins (22, 23, 41, 42, 46). Detailed structural studies will be necessary to fully elucidate the configuration by which FHL proteins contact other proteins.

We have found that FHL3 behaves as a potent transcriptional repressor of both basal and activated transcription when tethered to a promoter region either by the gal4DBD or by the zinc finger DBD of BKLF. Furthermore, FHL3 expression (without a linked DBD) can potentiate BKLF repression of GATA-1 activation. Taken together with the *in vitro* and *in vivo* binding data between BKLF and FHL3 presented above, this argues strongly that FHL3 functions as a BKLF co-repressor. This is further supported by recent observations that FHL3 (and FHL2) can also act as a co-repressor for the multi-zinc finger transcription factor PLZF (30). Because FHL3 binds CtBP, the transcriptional repression observed may be due, at least in part, to its ability to recruit one or more endogenous CtBP proteins to the promoter. In a previous study, FHL3 behaved as transcriptional activator (23). Differences in the context of the promoter are most likely to explain these different findings. We have chosen promoters that BKLF and CtBP2 can potently repress in cellular assays, and these may be particularly sensitive to FHL3. Differences in cell lines, and hence other accessory proteins, may also be important. Furthermore, in the context of recruitment by DNA-binding factors, unique interactions with different FHL3 target proteins may alter the conformation of FHL3, allowing it to interact with either co-activators or co-repressors through the presentation of distinct protein interaction domains.

FHL proteins are not exclusively nuclear and have been shown to bind both nuclear and cytoplasmic partner proteins (20–23, 41, 42, 46–48). Furthermore, sub-cellular partitioning of FHL proteins has been implicated in the regulation of their function. For example, Rho-signaling promotes the nuclear localization of FHL2, and subsequent co-activation of the AR (31), and the kinesin KIF17b binds ACT and is thought to counter co-activation of CREM by ACT by shuttling it from the nucleus to the cytoplasm (32). We have found that the subcellular localization of FHL3 is influenced by the availability of its cofactors. When FHL3 is expressed in COS cells on its own, or with either BKLF or CtBP2, it is primarily cytoplasmic. However, co-expression with both BKLF and CtBP2 markedly increases the amount of FHL3 (and CtBP2) found in the nucleus. Additionally, stable transgene expression of BKLF in erythroleukemia cells promotes the nuclear accumulation of endogenous FHL3 and CtBP2. Indeed, gel-filtration chromatography shows that a large complex of ~240 kDa containing BKLF, CtBP2, and FHL3 is found in the nucleus and not in the cytoplasm. The full composition of this complex is not certain. However, given the molecular weights of the three proteins, BKLF (at 39 kDa), FHL3 (at 31 kDa), and CtBP2 (at 49 kDa), and the knowledge that each can self-associate, the simplest explanation is that the complex contains a dimer of each component (totaling 239 kDa). The presence of a nuclear-specific complex containing BKLF, CtBP2, and FHL3 is supported by co-immunoprecipitation data, showing that BKLF in the nucleus binds both CtBP2 and FHL3. Cytoplasmic BKLF behaves very differently in gel filtration and, as judged by co-immuno-

precipitation studies, does not bind appreciable amounts of FHL3 or CtBP2. Post-translational modification of BKLF may be important in directing it to the nucleus and allowing it to associate with different partner proteins. Alternatively, BKLF, carrying a putative nuclear localization signal (49), may form a complex with CtBP2 and FHL3 in the cytoplasm, and this complex may then be shuttled to the nucleus. We have previously found that BKLF can behave as both a transcriptional activator (35)[2] and as a repressor (14). These intriguing results may be accounted for by the presence of different post-translational states and/or the availability of partners such as FHL3 and/or CtBP2.

Members of the Sp/KLF family, numbering greater than 20, share related DNA-binding domains composed of three Krüppel-like zinc fingers, and all bind to similar sites in DNA (1–4). Many of these proteins are co-expressed in various cell types, and a current challenge is to understand whether this co-expression facilitates redundant regulation of similar genes or regulation of specific genes. So far there is strong evidence that some KLF members, such as EKLF/KLF1, have specific target genes, like β-globin, that are not activated by other members of the family. The observation that different family members share little homology outside their DNA-binding domains and hence interact with distinct cofactors may be of central importance. Potentially, each KLF may interact with a unique set of cofactors that allows it to regulate a specific subset of genes. On this note, it has been shown that FHL3 does not bind the related KLF Sp1 (22). Thus, the complex we observe between BKLF, CtBP2, and FHL3 may be unique to BKLF, allowing it to repress a specific set of target genes. Future work correlating target genes with cofactor interactions, for example using chromatin immunoprecipitation with antibodies to a specific KLF and its cofactors, may afford insight into this complex problem.

Acknowledgments—We are grateful to members of the laboratory, and to Melissa Holmes, for careful reading of the manuscript and helpful suggestions.

REFERENCES

1. Turner, J., and Crossley, M. (1999) *Trends Biochem. Sci.* **24,** 236–240
2. Bieker, J. J. (2001) *J. Biol. Chem.* **276,** 34355–34358
3. Dang, D. T., Pevsner, J., and Yang, V. W. (2000) *Int. J. Biochem. Cell Biol.* **32,** 1103–1121
4. Philipsen, S., and Suske, G. (1999) *Nucleic Acids Res.* **27,** 2991–3000
5. Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R., and Grosveld, F. (1995) *Nature* **375,** 316–318
6. Perkins, A. C., Sharpe, A. H., and Orkin, S. H. (1995) *Nature* **375,** 318–322
7. Kuo, C. T., Veselits, M. L., Barton, K. P., Lu, M. M., Clendenin, C., and Leiden, J. M. (1997) *Genes Dev.* **11,** 2996–3006
8. Kuo, C. T., Veselits, M. L., and Leiden, J. M. (1997) *Science* **277,** 1986–1990
9. Wani, M. A., Wert, S. E., and Lingrel, J. B. (1999) *J. Biol. Chem.* **274,** 21180–21185
10. Armstrong, J. A., Bieker, J. J., and Emerson, B. A. (1999) *Cell* **95,** 93–104
11. Zhang, W., and Bieker, J. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9855–9860
12. Schuierer, M., Hilger-Eversheim, K., Dobner, T., Bosserhoff, A. K., Moser, M., Turner, J., Crossley, M., and Buettner, R. (2001) *J. Biol. Chem.* **276,** 27944–27949
13. Turner, J., and Crossley, M. (2001) *Bioessays* **23,** 683–690
14. Turner, J., and Crossley, M. (1998) *EMBO J.* **17,** 5129–5140
15. van Vliet, J., Turner, J., and Crossley, M. (2000) *Nucleic Acids Res.* **28,** 1955–1962
16. Zhang, C. L., McKinsey, T. A., Lu, Jr., and Olson, E. N. (2001) *J. Biol. Chem.* **276,** 35–39
17. Sundqvist, A., Sollerbrant, K., and Svensson, C. (1998) *FEBS Lett.* **429,** 183–188
18. Sewalt, R. G., Gunster, M. J., van der Vlag, J., Satijn, D. P., and Otte, A. P. (1999) *Mol. Cell. Biol.* **19,** 777–787
19. Koipally, J., and Georgopoulos, K. (2000) *J. Biol. Chem.* **275,** 19594–19602
20. Taniguchi, Y., Furukawa, T., Tun, T., Han, H., and Honjo, T. (1998) *Mol. Cell. Biol.* **18,** 644–654
21. Fimia, G. M., De Cesare, D., and Sassone-Corsi, P. (1999) *Nature* **398,** 165–169
22. Fimia, G. M., De Cesare, D., and Sassone-Corsi, P. (2000) *Mol. Cell. Biol.* **20,** 8613–8622
23. Muller, J. M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., Breyer, T., Holubarsch, C., Buettner, R., and Schule, R. (2000) *EMBO J.* **19,** 359–369
24. Morgan, M. J., Madgwick, A. J., Charleston, B., Pell, J. M., and Loughna, P. T. (1995) *Biochem. Biophys. Res. Commun.* **212,** 840–846
25. Morgan, M. J., and Madgwick, A. J. (1996) *Biochem. Biophys. Res. Commun.* **225,** 632–638
26. Morgan, M. J., and Madgwick, A. J. (1999) *Biochem. Biophys. Res. Commun.* **255,** 251–255
27. Bach, I. (2000) *Mech. Dev.* **91,** 5–17
28. Freyd, G., Kim, S. K., and Horvitz, H. R. (1990) *Nature* **344,** 876–879
29. Du, X., Hublitz, P., Gunther, T., Wilhelm, D., Englert, C., and Schule, R. (2002) *Biochim. Biophys. Acta* **1577,** 93–101
30. McLoughlin, P., Ehler, E., Carlile, G., Licht, J. D., and Schafer, B. W. (2002) *J. Biol. Chem.* **277,** 37045–37053
31. Muller, J. M., Metzger, E., Greschik, H., Bosserhoff, A. K., Mercep, L., Buettner, R., and Schule, R. (2002) *EMBO J.* **21,** 736–748
32. Macho, B., Brancorsini, S., Fimia, G. M., Setou, M., Hirokawa, N., and Sassone-Corsi, P. (2002) *Science* **298,** 2388–2390
33. Crossley, M., Merika, M., and Orkin, S. H. (1995) *Mol. Cell. Biol.* **15,** 2448–2456
34. Crossley, M., Tsang, A. P., Bieker, J. J., and Orkin, S. H. (1994) *J. Biol. Chem.* **269,** 15440–15444
35. Crossley, M., Whitelaw, E., Perkins, A., Williams, G., Fujiwara, Y., and Orkin, S. H. (1996) *Mol. Cell. Biol.* **16,** 1695–1705
36. Konopka, J. B., Watanabe, S. M., and Witte, O. N. (1984) *Cell* **37,** 1035–1042
37. Dean, A., Ley, T. J., Humphries, R. K., Fordis, M., and Schechter, A. N. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5515–5519
38. Perkins, A. C., Yang, H., Crossley, P. M., Fujiwara, Y., and Orkin, S. H. (1997) *Blood* **90,** Suppl. 1, 575
39. Turner, J., and Crossley, M. (1999) *Int. J. Biochem. Cell Biol.* **31,** 1169–1174
40. Li, H. Y., Ng, E. K., Lee, S. M., Kotaka, M., Tsui, S. K., Lee, C. Y., Fung, K. P., and Waye, M. M. (2001) *J. Cell. Biochem.* **80,** 293–303
41. Wixler, V., Geerts, D., Laplantine, E., Westhoff, D., Smyth, N., Aumailley, M., Sonnenberg, A., and Paulsson, M. (2000) *J. Biol. Chem.* **275,** 33669–33678
42. Ng, E. K., Chan, K. K., Wong, C. H., Tsui, S. K., Ngai, S. M., Lee, S. M., Kotaka, M., Lee, C. Y., Waye, M. M., and Fung, K. P. (2002) *J. Cell. Biochem.* **84,** 556–566
43. Zon, L. I., Youssoufian, H., Mather, C., Lodish, H. F., and Orkin, S. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10638–10641
44. Martin, D. I., Tsai, S. F., and Orkin, S. H. (1989) *Nature* **338,** 435–438
45. Kumar, V., Carlson, J. E., Ohgi, K. A., Edwards, T. A., Rose, D. W., Escalante, C. R., Rosenfeld, M. G., and Aggarwal, A. K. (2002) *Mol. Cell* **10,** 857–869
46. Chan, K. K., Tsui, S. K., Ngai, S. M., Lee, S. M., Kotaka, M., Waye, M. M., Lee, C. Y., and Fung, K. P. (2000) *J. Cell. Biochem.* **76,** 499–508
47. Amaar, Y. G., Thompson, G. R., Linkhart, T. A., Chen, S. T., Baylink, D. J., and Mohan, S. (2002) *J. Biol. Chem.* **277,** 12053–12060
48. Dye, B. T., and Patton, J. G. (2001) *Exp. Cell Res.* **263,** 131–144
49. Shields, J. M., and Yang, V. W. (1997) *J. Biol. Chem.* **272,** 18504–18507

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 26, Issue of June 27, pp. 24139–24152, 2003
Printed in U.S.A.

FHL3 Is an Actin-binding Protein That Regulates α-Actinin-mediated Actin Bundling

FHL3 LOCALIZES TO ACTIN STRESS FIBERS AND ENHANCES CELL SPREADING AND STRESS FIBER DISASSEMBLY*

Received for publication, December 30, 2002, and in revised form, March 20, 2003
Published, JBC Papers in Press, April 18, 2003, DOI 10.1074/jbc.M213259200

Imogen D. Coghill, Susan Brown‡, Denny L. Cottle, Meagan J. McGrath§, Paul A. Robinson, Harshal H. Nandurkar, Jennifer M. Dyson, and Christina A. Mitchell¶

From the Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Melbourne, Victoria, Australia

Four and a half LIM domain (FHL) proteins are members of the LIM protein superfamily. Several FHL proteins function as co-activators of CREM/CREB transcription factors and the androgen receptor. FHL3 is highly expressed in skeletal muscle, but its function is unknown. FHL3 localized to the nucleus in C2C12 myoblasts and, following integrin engagement, exited the nucleus and localized to actin stress fibers and focal adhesions. In mature skeletal muscle FHL3 was found at the Z-line. Actin was identified as a potential FHL3 binding partner in yeast two-hybrid screening of a skeletal muscle library. FHL3 complexed with actin both *in vitro* and *in vivo* as shown by glutathione *S*-transferase pull-down assays and co-immunoprecipitation of recombinant and endogenous proteins. FHL3 promoted cell spreading and when overexpressed in spread C2C12 cells disrupted actin stress fibers. Increased FHL3 expression was detected in highly motile cells migrating into an artificial wound, compared with non-motile cells. The molecular mechanism by which FHL3 induced actin stress fiber disassembly was demonstrated by low speed actin co-sedimentation assays and electron microscopy. FHL3 inhibited α-actinin-mediated actin bundling. These studies reveal FHL3 as a significant regulator of actin cytoskeletal dynamics in skeletal myoblasts.

The LIM superfamily of proteins is defined by the presence of one or more LIM domains, which represent a cysteine-rich double zinc finger motif denoted by the sequence ($CX_2CX_{17-19}HX_2C)X_2(CX_2CX_{16-20}CX_2$(H/D/C)) (1). The LIM zinc finger does not interact with DNA but functions as a protein-protein binding module (2, 3). LIM proteins localize to the nucleus and scaffold the assembly of transcription factors and thereby regulate transcription. In the cytoplasm LIM proteins facilitate the complex association of signaling proteins with the actin cytoskeleton (3).

A subset of LIM proteins are the LIM-only proteins, which comprise solely multiple copies of LIM domains. The family of four and a half LIM domain (FHL)[1] proteins all contain a single N-terminal LIM-type zinc finger followed by four sequential LIM domains and no other modular domains (4). This group of proteins includes FHL1 (also called SLIM1) and its alternatively spliced isoforms SLIMMER and KyoT2, FHL2 (also called DRAL for "down-regulated in rhabdomyosarcoma" or SLIM3), FHL3 (also called SLIM2), FHL4, and ACT. FHL1, FHL2, and FHL3 are highly expressed in striated muscle, whereas ACT is expressed only in the testis (4–12).

FHL1 is strongly implicated in the pathogenesis of human cardiomyopathy. Microarray analysis has demonstrated FHL1 mRNA levels are decreased in failing human hearts with dilated cardiomyopathy and significantly increased in hypertrophic cardiomyopathy (13, 14). Mouse models of hypertrophic cardiomyopathy induced by transverse aortic constriction also show elevated expression of FHL1, suggesting FHL1 may play a significant role in regulating the heart muscle cytoarchitecture (15). In skeletal myoblasts FHL1 localizes in an integrin-dependent manner to the nucleus, focal adhesions, and stress fibers (9, 16). To date no protein binding partners have been identified that associate with FHL1 in either the nucleus or cytoskeleton.

FHL2 is expressed in cardiac but not skeletal muscle and acts as a transcriptional co-activator for the CREB/CREM transcription factors and the androgen receptor (17–19). FHL2 binds a variety of receptors and signaling and structural proteins including the cytoplasmic domains of α and β integrins, insulin-like growth factor receptor-binding protein 5, the Alzheimer's disease-associated protein presenilin-2, the promyelocytic leukemia zinc finger protein (PLZF), and the transcription factor WT1; in addition, FHL2 homodimerizes and forms heterodimers with FHL3 (20–25). Recently, FHL2 has been shown to complex with the metabolic enzymes creatinine kinase, adenylate kinase, and phosphofructokinase (26). Interestingly, FHL2 has been shown by two groups to interact with β-catenin and regulate β-catenin T-cell factor-mediated transcriptional events (27, 28). Mice with targeted deletion of FHL2 develop normally but demonstrate enhanced cardiac hypertrophy in response to β-adrenergic stimulation (29, 30).

The least characterized protein of the FHL family, FHL3, is

* This work was supported in part by a grant from the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a National Heart Foundation postdoctoral fellowship.
§ Recipient of a National Heart Foundation postgraduate research scholarship.
¶ To whom correspondence should be addressed. Fax: 61-3-9905-4699; E-mail: christina.mitchell@med.monash.edu.au.

[1] The abbreviations used are: FHL, four and a half LIM domains; AD, GAL-4 activation domain; BD, GAL-4 DNA-binding domain; DTT, dithiothreitol; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; IPTG, isopropylthiogalactosidase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; CREB, cAMP-response element-binding protein; TMRM, tetramethylrhodamine methyl ester perchlorate; CREM, cAMP response element modulator.

TABLE I
Oligonucleotides used for the generation of FHL3 and actin constructs

Name of construct	5′ Oligonucleotide	3′ Oligonucleotide	Polypeptide expressed
FHL3	5′-ggcacgagcccgttcgagggtt-3′	5′-gagctgagtcccagaggtggt-3′	Used as template for subsequent cloning
FHL3-*Eco*RI	5′-tctgaattcatgagcgagtcatttgactgt-3′	3′-tctgaattcttagggccctgcctggctaca-3′	Full-length FHL3 (aa[a] 1-280)
FHL3-*Xba*I	5′-tcttctagaatgagcgagtcatttgactgt-3′	5′-tcttctagattagggccctgcctggctaca-3′	Full-length FHL3 (aa 1-280)
FHL3 LIM 1/2, 1 and 2	5′-tgtgaattcatgagcgagtcatttgactgtgca-3′	5′-tgtgaattcttagcgaggagcaaacttgttctc-3′	The 1/2, first and second LIM domains (aa 1-161)
FHL3 LIM 2 and 3	5′-tgtgaattctactgcagtgcgttttcctcg-3′	5′-tgtgaattcttacttaggtgcaaagagttctcc-3′	The second and third LIM domains (aa 93-220)
FHL3 LIM 3 and 4	5′-tgtgaattctatgagaacaagtttgctcctcgc-3′	5′-tgtgaattcttagggccctgcctggctaca-3′	The third and fourth LIM domains (aa 154-280)
α-Skeletal actin	5′-tctgaattcacgcgtatgtgcgacgaagac-3′	5′-tctgaattcacgcgttaagaagcatttgcg-3′	Full length α-skeletal actin (aa 1-377)

[a] aa, amino acids.

the focus of this study. Previous studies (22) have shown FHL3 localizes to the nucleus and focal adhesions in C2C12 myoblasts. Consistent with its nuclear localization, and like FHL2, FHL3 acts as a co-activator for the transcription factor CREB, independent of the CREB-binding protein, thus providing a mechanism for CREB-mediated transcription (10, 18). In addition, FHL3 strongly activates a GAL-driven luciferase reporter, in response to Rho-GTPase activation (31). As Rho activation regulates actin cytoskeletal dynamics, the localization of FHL3 to the nucleus and focal adhesions is consistent with a transcriptional role for FHL3, downstream of Rho signaling (32). Given FHL3 contains four and a half LIM domains, this protein has the potential to scaffold the assembly of many proteins in either the nucleus or cytoplasm. However, the physiological function of FHL3 in skeletal muscle is unknown.

In this study we have demonstrated that integrin engagement regulates FHL3 re-localization from the nucleus to actin stress fibers in myoblasts. By using yeast two-hybrid screening of a skeletal muscle library, we have identified actin as an FHL3 binding partner. The interaction between FHL3 and actin has been confirmed both *in vitro* and *in vivo*. FHL3 regulates stress fibers by inhibiting α-actinin-mediated actin bundling. Thus, in addition to regulating transcription downstream of Rho activation (31), FHL3 regulates actin bundling and thereby actin stress fiber remodeling.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were obtained from New England Biolabs, Fermentas, or Promega. Big Dye Terminator cycle sequencing was from PE Applied Systems. The yeast two-hybrid Matchmaker 3 system and the pEGFP-C2 vector were obtained from Clontech. Oligonucleotides were purchased from the Department of Microbiology, Monash University, Melbourne, Australia. C2C12 and COS-1 cells were from American Tissue Type Collection. Dulbecco's modified Eagle's medium was obtained from Trace Biosciences; fetal calf serum was from Commonwealth Serum Laboratories, and horse serum from Invitrogen. Chiron Mimotopes generated synthetic peptides. Monoclonal antibodies to FLAG and α-actinin were obtained from Sigma. Polyclonal rabbit α-actinin and goat actin antibodies were obtained from Santa Cruz Biotechnology. Hemagglutinin (HA) monoclonal antibodies were obtained from Silenus. Texas Red phalloidin was purchased from Molecular Probes, and propidium iodide was from Sigma. All other reagents were from Sigma unless otherwise specified. The α-skeletal-actin cDNA was a gift from Dr. Edna Hardeman (Children's Medical Research Institute, Sydney, Australia). The pCGN vector was a gift from Dr. Tony Tiganis (Monash University, Melbourne, Australia). The pEFBOS-FLAG vector was a gift from Dr. Tracey Wilson (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The pGEX-5X-1 vector was from Amersham Biosciences.

Antipeptide Antibodies—The first and last 6 amino acids of FHL3 were fused together to generate the peptide sequence "MSESFDCSQAGP" and linked to diphtheria toxin at the central cysteine residue. This conjugated peptide was injected subcutaneously into two New Zealand White rabbits. FHL3 antibodies were purified by affinity chromatography from preimmune or immune sera on an FHL3 peptide-coupled thiopropyl-Sepharose resin and eluted in 0.1 M glycine HCl, pH 2.5.

Immunoblot Analysis of Recombinant FHL Proteins—COS-1 cells were transfected with HA-FHL1, HA-FHL2, or HA-FHL3 by electroporation using 5 μg of DNA. Following transfection, cells were rested for 24–36 h before harvesting. Cells were washed with Tris-buffered saline and lysed with Tris-buffered saline, 1% Triton X-100, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin for 2 h at 4 °C. Lysates were centrifuged at 16,000 × *g* for 20 min, and the soluble fraction was analyzed by SDS-PAGE and immunoblotted with antibodies to FHL3 or HA. In some studies the C2C12 myoblast cell line (CRL-1772) was grown at low confluence (50%) in growth media (Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1% streptomycin). Differentiation into myotubes was induced by switching cells to differentiation media (Dulbecco's modified Eagle's medium supplemented with 5% horse serum, 2 mM L-glutamine, 100 units/ml penicillin, and 0.1% streptomycin) for a further 24–120 h. Cells were harvested and lysates immunoblotted for FHL3.

Generation of Full-length FHL3 and FHL3 Truncation Mutants—The full-length FHL3 cDNA was cloned from Marathon Ready skeletal muscle cDNA (Clontech) using a touchdown PCR protocol. Further PCR was performed to introduce specific restriction sites at the 5′ and 3′ ends of the full-length clone. Full-length FHL3 was cloned in-frame into pEGFP-C2 (*Eco*RI site), pCGN (*Xba*I site), and pGEX-5X1 (*Eco*RI site) generating the N-terminal FHL3 fusion proteins with green fluorescent protein (GFP), hemagglutinin (HA), and glutathione *S*-transferase (GST), respectively. Truncation mutants of FHL3 were generated with 5′ and 3′ *Eco*RI restriction sites by PCR and cloned into the yeast two-hybrid Matchmaker 3 bait vector pGBKT7 vector via the *Eco*RI site creating N-terminal GAL-4 fusion "bait" proteins. All PCR products and constructs were verified by dideoxy sequencing in both directions. Oligonucleotides used to generate these constructs are presented in Table I.

Intracellular Localization of FHL3 in C2C12 Myoblasts—C2C12 cells were left untransfected or transfected with either HA-FHL3 or HA-β-galactosidase, using LipofectAMINE as per manufacturer's instructions (Invitrogen). C2C12 cells were plated in 6-well dishes at 4 × 10^5 cells/well onto fibronectin (5 μg/ml)-coated glass coverslips for 1 or 3 h. To determine factors mediating FHL3 localization, HA-FHL3-transfected C2C12 myoblasts were plated onto fibronectin or poly-L-lysine (1 mg/ml)-coated glass coverslips for 60 or 180 min at 37 °C. In the leptomycin B experiments, HA-FHL3- transfected cells were plated onto fibronectin-coated coverslips and placed into media containing vehicle or leptomycin B (2 ng/ml) for 60 or 180 min at 37 °C. To determine the effect of cytochalasin D treatment on FHL3 localization, GFP-FHL3-transfected C2C12 cells were plated onto fibronectin-coated coverslips for 3 h at 37 °C and then placed into media containing either vehicle (Me_2SO), or cytochalasin D (5 mM) for 30 min at 37 °C. Cells were fixed and permeabilized with PBS, 3.7% paraformaldehyde, 0.2% Triton X-100 for 10 min at room temperature and blocked with 1% bovine serum albumin (BSA) in PBS for 10 min. Cells were stained with anti-FHL3 or anti-HA antibodies (1:5000) for 1 h at room temperature. FHL3 antibodies were detected with FITC anti-rabbit IgG and anti-HA antibodies with FITC anti-mouse IgG for 1 h at room temperature. Co-localization was performed with the F-actin stain Texas Red phalloidin, the nuclear stain propidium iodide, or anti-paxillin antibodies. In some experiments cells were triple-labeled with anti-HA (detected with anti-rabbit Cy5), Texas Red phalloidin, and anti-paxillin antibodies. Samples were mounted on glass slides using SlowFade and viewed using confocal microscopy.

In some studies to assess cell viability, HA-FHL3 or HA-β-galactosidase-transfected cells were labeled with tetramethylrhodamine methyl ester perchlorate (TMRM) and propidium iodide and added to the media for 5 min. Live cells were then immediately imaged by confocal microscopy and were considered viable if propidium iodide was excluded from the nucleus, and the mitochondrial dye TMRM was taken up by the cell and mitochondrial fluorescence detected. Cells from the same transfection were fixed and stained with anti-HA antibodies to determine transfection efficiency.

Intracellular Localization of FHL3 in Mouse Skeletal Muscle Sections—Mice were killed following the guidelines of the National Health and Medical Research Council, Monash University animal ethics number BAM/2000/17. The soleus muscle was dissected from the hind limbs of the mice and snap-frozen in isopentane at resting length. Muscles were placed in OCT blocks and cryosectioned at −20 °C in 7-μm longitudinal and transverse sections. Sections were placed onto Superfrost Plus glass slides and fixed in phosphate-buffered saline (PBS), 4% paraformaldehyde for 5 min at room temperature. Samples were blocked and permeabilized for 15 min with PBS, 10% horse serum, 0.1% Triton X-100. Sections were washed with PBS and incubated with primary antibodies overnight at 4 °C (33). The primary antibodies used were affinity-purified FHL3 antibodies, α-actinin antibodies at 1:800, and actin antibodies at 1:600. Detection of primary antibodies with secondary antibodies was as follows. FHL3 antibodies were detected with FITC anti-rabbit IgG; α-actinin antibodies were detected with TRITC anti-mouse IgG, and actin antibodies were detected with TRITC anti-goat IgG. Sections were incubated with secondary antibodies for 2 h and then washed with PBS. Sections were mounted using SlowFade and visualized using confocal microscopy.

Yeast Two-hybrid Analysis—The Matchmaker 3 GAL-4-based yeast two-hybrid system was used. The yeast strain AH109 was transformed with the plasmid encoding the fusion between the GAL4 DNA binding domain (BD) fused with the N-terminal FHL3 LIM domain bait construct (BD-1/2, -1, and -2). Yeast expressing BD-1/2, -1, and −2 were transformed with a human skeletal muscle cDNA library fused to the GAL-4 activation domain (AD) as per the manufacturer's instructions (Clontech). Plasmids from positive clones were extracted as described previously (34). Y187 yeast, an opposing mating strain of AH109 yeast, was transformed with the AD-α-skeletal actin expressing plasmid (encoding amino acids 205–377) to investigate FHL3-actin interactions. AH109 yeast expressing bait plasmids of the LIM domains from FHL1, FHL2, FHL3, and KyoT2 were mated with Y187 yeast expressing the AD-actin construct and plated onto selective media as per the manufacturer's instructions (Clontech).

Generation of Full-length Actin Constructs—PCR was performed to add *Mlu*I and *Eco*RI restriction sites to the 5′ and 3′ ends of the cDNA encoding the open reading frame of α-skeletal-actin cDNA, (see Table I for oligonucleotides). Subsequently actin was cloned in-frame into pEF-BOS(Flag) at the *Mlu*I site.

In Vitro GST Pull-down Assays—GST alone or the GST-FHL3 fusion protein was expressed in *Escherichia coli* by growing the transformed cells at 37 °C to log phase (A_{600} of 0.6). Fusion protein expression was induced by 100 μM isopropylthiogalactosidase, and cells were incubated at 25 °C for 16 h. Induced cells were lysed with PBS, 1% Triton X-100, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin for 2 h at 4 °C. Lysates were cleared and purified on glutathione-Sepharose 4B (Amersham Biosciences) for 30 min at room temperature (35). After extensive washing, fusion proteins were eluted using 10 mM reduced glutathione in a buffer containing 50 mM Tris-HCl, pH 8.0, and 0.5 mM dithiothreitol (DTT). GST-FHL3 proteins were washed to remove glutathione and concentrated using a vivaspin 50,000 molecular weight cut-off concentrator. 0.5 μmol of GST or GST-FHL3 was bound to 10 μl of glutathione-Sepharose resin in 100 μl of XB buffer (10 mM Hepes, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.1% (v/v) Tween 20, and 0.2 mM ATP) for 30 min at room temperature. The resin was washed once with XB buffer. 4 μM actin in 100 μl of XB buffer was added to the resin and incubated for 30 min at room temperature. The resin was washed 3 times with XB buffer and boiled in SDS sample reducing buffer for 5 min and immunoblotted with GST or actin antibodies.

Co-immunoprecipitation of Recombinant and Endogenous Actin and FHL3—Untransfected C2C12 myoblasts or COS-1 cells co-transfected with HA-FHL3 and FLAG-actin were harvested in actin lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 300 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Cells were lysed for 1 h at 4 °C, and the C2C12 soluble lysate was mixed with non-immune sera or anti-FHL3 antibodies and the COS-1 lysates with non-immune sera or 10 μg of FLAG antibodies and protein A-Sepharose for 2 h at 4 °C. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with FHL3 or actin antibodies (C2C12 cells) or FLAG and HA antibodies (COS-1 cells).

Cell Spreading Assays—C2C12 cells transfected with GFP vector alone or GFP-FHL3 were plated at a density of 4×10^5 cells/well onto fibronectin (5 μg/ml)-coated coverslips in 6-well plates. Cells were allowed to spread for 15, 60, or 180 min before fixation and permeabilization (16). Fixed cells were stained with propidium iodide to identify the nucleus. Cells were considered spread if the cytoplasmic surface area was at least twice the nuclear surface area. 100 cells on each coverslip were counted and scored for spreading in three separate experiments of three independent transfections.

Low Speed Actin Co-sedimentation Assays—Purified rabbit soleus muscle actin (16 μM) stored in Ca-ATP buffer (2 mM imidazole, 0.1 mM $CaCl_2$, 0.5 mM DTT and 0.2 mM ATP) was polymerized by the addition of 1/10 volume of exchange buffer (10 mM EDTA and 1 mM $MgCl_2$) for 10 min at room temperature, followed by the addition of 1/10 volume of 1 M KCl for 1 h at room temperature. The actin mixtures were then diluted 1/4 with Mg-ATP buffer (2 mM imidazole, 0.1 mM $MgCl_2$, 0.5 mM DTT and 0.2 mM ATP) in the presence of purified α-actinin (250 nM) and/or purified GST (250 nM) or GST-FHL3 (250 nM) in a final reaction volume of 50 μl. The actin mixtures were pelleted by centrifugation at $10,000 \times g$ for 15 min. The supernatant and the pellet were separated and analyzed by 12.5% SDS-PAGE, and proteins were detected by Coomassie Brilliant Blue staining or immunoblotting with actin antibodies. Densitometry was performed on Coomassie-stained actin pellets using a UMAX Astra 1220U scanner and GelPro software in three separate experiments.

Electron Microscopy—Actin (16 μM) stored in Ca-ATP buffer was polymerized by the addition of 1/10 volume of exchange buffer for 10 min at room temperature, followed by the addition of 1/10 volume of 1 M KCl for 1 h at room temperature. The actin mixtures were then diluted 1/8 with Mg-ATP buffer in the presence of purified α-actinin (500 nM) alone or with α-actinin and either purified GST (500 nM) or GST-FHL3 (500 nM), in a final reaction volume of 25 μl. These mixtures were incubated for 1 h at room temperature. The protein mixtures were adsorbed onto carbon-coated 400 mesh grids for 1 min. Actin filaments were negatively stained with 2% phosphotungstic acid, pH 7.4, for 15 s. Grids were visualized using transmission electron microscopy (model H-5700, Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV and a nominal magnification of ×100,000.

Wounding Assay—C2C12 cells were plated to 100% confluence onto coverslips coated with fibronectin (5 μg/ml) and wounded with a plastic pipette. The wound was allowed to close for 24 h, prior to fixation and staining with anti-FHL3 antibodies or phalloidin staining (36).

RESULTS

Generation of Anti-peptide Antibodies to FHL3—FHL3 is highly expressed in skeletal muscle (6, 12). To characterize FHL3 in myoblasts and skeletal muscle, anti-peptide antibodies were generated to FHL3 sequence derived from the first six N-terminal and the last six C-terminal amino acids, which were conjugated to diphtheria toxin at the central cysteine residue (Fig. 1*A*). The human FHL3 amino acid sequence used as a target for antibody production demonstrates 92% identity with mouse FHL3 sequence, 40% identity with FHL1 and FHL2, and less than 20% with ACT. To demonstrate specificity of the FHL3 anti-peptide antibody, COS-1 cells were transiently transfected with HA-tagged constructs encoding FHL1, FHL2, or FHL3, and lysates were analyzed by immunoblot analysis, using either FHL3 antipeptide antibodies (Fig. 1*B, upper panel*) or HA monoclonal antibodies (Fig. 1*B, lower panel*). The HA antibody detected recombinant HA-tagged FHL1, FHL2, and FHL3, migrating at the predicted molecular mass of ~36 kDa. Probing these same lysates with anti-peptide antibodies specific to FHL3 demonstrated an immunoreactive 36-kDa polypeptide only in FHL3-transfected cells. Affinity-purified FHL3 antipeptide antibodies also detected a 34-kDa polypeptide in immunoblots of the Triton X-100-soluble fraction of adult mouse skeletal muscle lysates, consistent with FHL3 expression (Fig. 1*C*). Pre-immune sera showed no immunoreactivity against mouse skeletal muscle lysates (data not

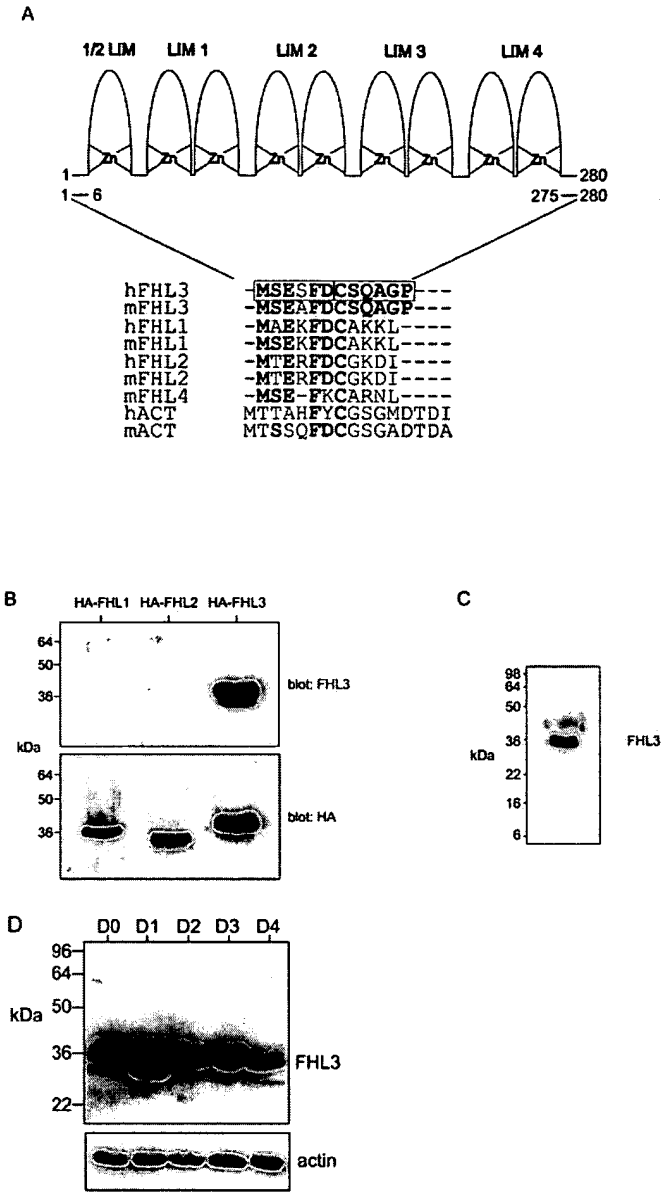

FIG. 1. **FHL3 antibody specificity.** *A, schematic* illustrating the LIM domains of FHL3. The N- and C-terminal FHL3 amino acid sequence used to raise the anti-peptide antibodies (in *box*) with an alignment of the corresponding sequence from the other FHL proteins is shown. Identical residues are shown in *boldface type. B,* COS-1 cells were transfected with HA-FHL1, HA-FHL2, or HA-FHL3. Triton-soluble lysates were analyzed by SDS-PAGE and immunoblotted using either affinity-purified FHL3 anti-peptide antibodies (*upper panel*) or HA antibodies (*lower panel*). *C,* mouse skeletal muscle was harvested and the Triton X-100-soluble lysate (100 μg) analyzed by SDS-PAGE and immunoblotted using FHL3 anti-peptide antibodies. The migration of molecular weight markers is shown on the *left* of *B* and *C. D,* C2C12 myoblasts were induced to differentiate for 4 days (*D 0–4*) as described under "Experimental Procedures." Cells were harvested daily and lysates immunoblotted for FHL3 (*upper panel*) and actin (*lower panel*).

shown). The level of FHL3 expression remained relatively constant as C2C12 myoblasts differentiated into myotubes, as assessed by immunoblot analysis (Fig. 1*D*).

FHL3 Localizes to the Nucleus and Stress Fibers in C2C12 Myoblasts—To determine the localization of FHL3 in skeletal myoblasts, undifferentiated C2C12 cells were plated onto coverslips coated with fibronectin, a ligand for the $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrin receptors which forms part of the extracellular matrix surrounding skeletal muscle (37, 38). Cells were fixed at 1 and 3 h after plating on fibronectin and stained with FHL3 anti-peptide antibodies and imaged using confocal microscopy (Fig. 2*A*). At 1 h after plating cells on fibronectin, FHL3 was detected in both the nucleus and cytoplasm (Fig. 2*A, i–iii*). FHL3 nuclear expression was confirmed by co-localization with PI. Imaging at the base of the adherent cell demonstrated FHL3 localized to focal adhesions (see *arrow* in *vi* and *ix*) and faintly at actin stress fibers, as shown by co-localization with phalloidin which stains polymerized actin, and α-actinin which localizes to actin stress fibers and at focal adhesions (Fig. 2*A, iv–vi* and *vii—ix*, respectively). Three hours after plating, the intensity of FHL3 staining in the nucleus had decreased (Fig. 2*A, x–xii*) and the intensity of FHL3 staining along actin stress fibers increased (Fig. 2*A, xiii–xv*). It is noteworthy that the co-localization of FHL3 with both phalloidin staining and α-actinin appeared in a patchy striated pattern along the actin fiber (Fig. 2*A,* see *arrowhead* in *vi, xv,* and *xviii*). FHL3 was also present at focal adhesions.

To corroborate the intracellular localization of FHL3 at actin stress fibers, which has not been previously reported, recombinant FHL3 was expressed in-frame with an N-terminal HA tag (Fig. 2*B*) or green fluorescent protein (GFP) (not shown) in C2C12 myoblasts. For the purposes of localization studies, only the low to moderate FHL3-expressing cells are shown. The high FHL3-expressing cells demonstrated cytoskeletal changes that will be discussed later. Three hours after plating, recombinant HA-FHL3 localized to actin stress fibers, as shown by co-localization with α-actinin. Consistent with the localization of endogenous FHL3, co-localization of HA-FHL3 with α-actinin was discontinuous (Fig. 2*B, i–iii*). The high magnification overlay image shows that HA-FHL3 localizes in a striated pattern along the actin stress fibers with bands of alternating α-actinin alone (*red*), HA-FHL3 alone (*green*), and joint α-actinin and HA-FHL3 (*yellow*) (Fig. 2*B, vi*). The same localization was observed using GFP-FHL3, indicating the N-terminal tag did not influence recombinant FHL3 localization (results not shown). We also co-localized HA-FHL3 with paxillin (39), a specific focal adhesion protein, and we demonstrated HA-FHL3 localized at focal adhesions (Fig. 2*B, vii–xii*).

FHL3 Nuclear Export Is Regulated by Integrin Activation and the CRM1 Export Pathway—Many actin-associated proteins shuttle between the cytoplasm and nucleus continuously, whereas for other proteins nuclear accumulation occurs only following specific cell stimuli. For example cell adhesion regulates the nuclear-cytoplasmic trafficking of members of the mitogen-activated protein kinase family, extracellular signal-regulated protein kinase and p38, c-Abl, and FHL1 (16, 40–43). Several recent studies (44–46) have demonstrated that LIM domain proteins such as zyxin and paxillin may relay information from adhesion sites to the nucleus. The re-plating of cells in suspension onto fibronectin-coated plates triggers the activation and clustering of integrin receptors, recruitment of adhesion complexes, and formation of focal adhesions, which in turn initiates actin polymerization at adhesion sites (47). When cells expressing HA-FHL3 were plated onto fibronectin (Fig. 3) or laminin (data not shown), 1 h after plating FHL3 demonstrated a dual localization in the nucleus and at focal adhesions (Fig. 3, *i*). However, following 3 h of plating on fibronectin (Fig. 3, *ii*) or laminin (results not shown), consistent with the localization of the endogenous protein, HA-FHL3 staining was predominantly cytoskeletal, strongly localizing to actin stress fibers, with minimal nuclear staining observed. Thus, following integrin activation, during cell spreading, FHL3 exits the nucleus and associates with the cytoskeleton. The exit of FHL3 from the nucleus occurred using either fibronectin or laminin as a matrix, which respectively activate the $\alpha_5\beta_1$, $\alpha_4\beta_1$, and $\alpha_7\beta_1$ integrin receptors (38, 48, 49), indicating FHL3 nuclear

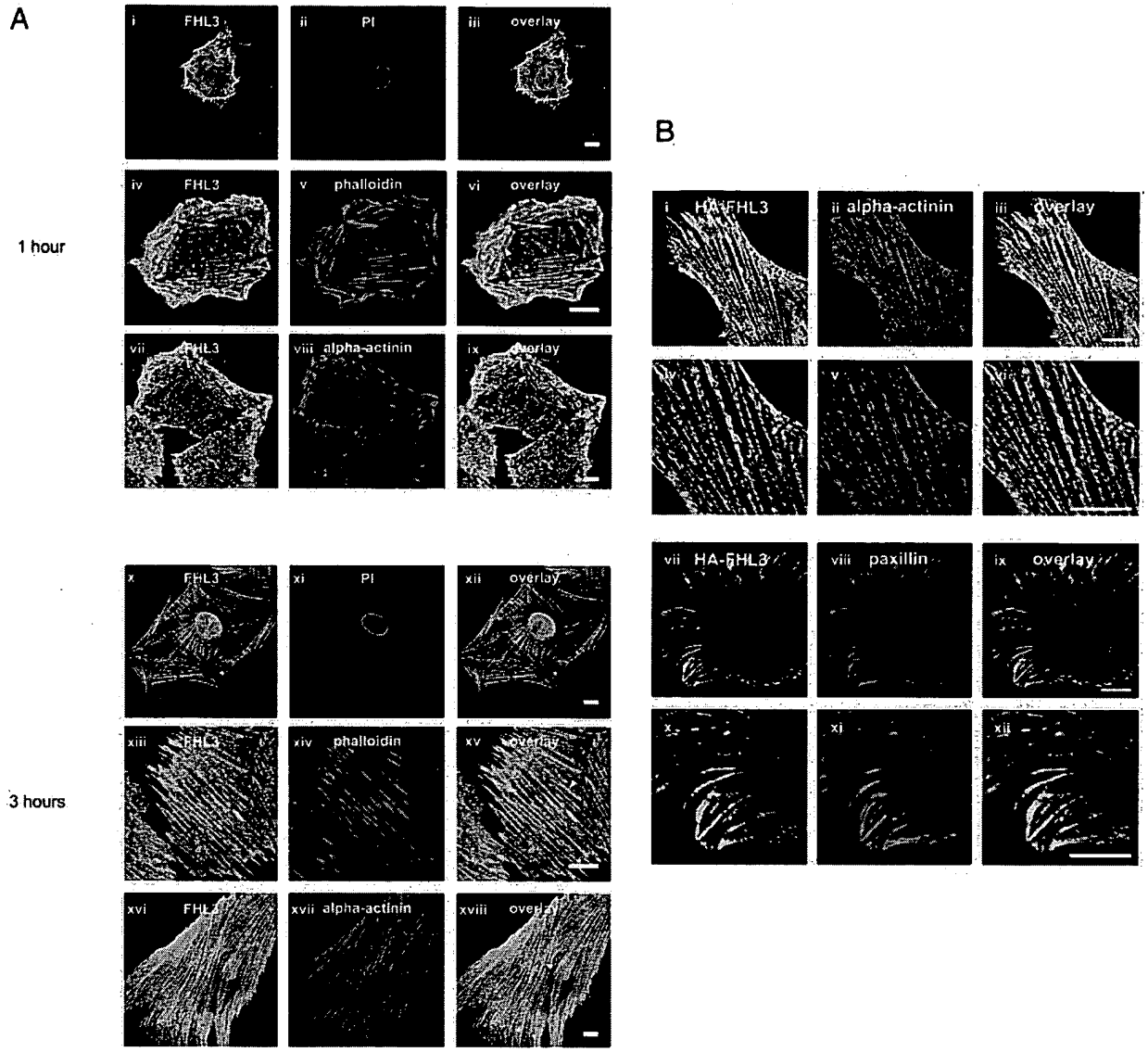

FIG. 2. **FHL3 localizes to the nucleus and actin stress fibers in C2C12 myoblasts.** *A*, C2C12 myoblasts were plated onto fibronectin-coated coverslips and fixed at 1 (*i–ix*) or 3 h (*x–xviii*) after plating. Cells were stained with FHL3 anti-peptide antibodies (*green, i, iv, vii, x, xiii,* and *xvi*) and co-stained with propidium iodide (*red, ii* and *xi*), or phalloidin (*red, v,* and *xiv*), or α-actinin antibodies (*red, viii* and *xvii*) and imaged by confocal microscopy. Overlay of the double immunofluorescence images are shown in the *right-hand panels. Arrow* in *vi* and *ix* indicates focal adhesion staining; *arrowhead* in *vi, xv,* and *xviii* indicates patchy co-localization of FHL3 with actin and α-actinin along actin stress fibers. *B*, C2C12 cells were transiently transfected with HA-FHL3, plated onto fibronectin-coated coverslips for 3 h, and fixed. Cells were stained with HA antibodies (*green, i, iv, vii,* and *x*) and co-stained with α-actinin antibodies (*red, ii* and *v*), or paxillin antibodies (*red, viii* and *xi*) and analyzed by confocal microscopy. Overlay of the double immunofluorescence images are shown on the *right. Scale bars* indicate 10 μm.

export relies on integrin activation and cell spreading but not activation through a specific integrin receptor. To determine whether integrin activation was required for FHL3 nuclear export, cells were plated onto poly-L-lysine, a substrate that permits cell adhesion but inhibits integrin activation (50). Cells plated on poly-L-lysine do not spread as integrin receptors are not activated. Under these conditions FHL3 demonstrated prominent nuclear localization and only faint cytoplasmic staining at both 1 and 3 h after plating on a fibronectin matrix (Fig. 3, *iii* and *iv,* respectively). Thus integrin activation may stimulate FHL3 nuclear export and relocation to the actin cytoskeleton.

We tested whether FHL3 nuclear export was dependent on the classical CRM1-dependent nuclear export pathway, which can be specifically inhibited using the fungal metabolite leptomycin B (51). CRM1 recognizes lysine-rich sequences in proteins and targets them for nuclear export (52, 53); although FHL3 lacks this motif, it may bind a protein that exits the nucleus via this pathway. Many cytoskeletal proteins, including actin, show a dual localization in the nucleus and the cytoskeleton and utilize this export pathway. Following leptomycin B treatment, after 1 h plating on fibronectin, HA-FHL3 localized in the nucleus and at focal adhesions (Fig. 3, *v*). After 3 h of plating cells on fibronectin in leptomycin B-treated cells, HA-FHL3 demonstrated prominent nuclear localization and little localization at actin stress fibers (Fig. 3, *vi*). The localization of FHL3 in leptomycin B-treated cells was quite distinct from the prominent stress fiber localization and absent nuclear expression of FHL3 in untreated cells on fibronectin at 3 h (Fig. 3, *ii*). Thus, leptomycin B treatment inhibited export of FHL3 from the nucleus to actin stress fibers. Although FHL3 has no lysine-rich nuclear export sequence, it is possible that FHL3 interacts with a protein that contains a lysine-rich export sequence and can thus co-transport FHL3 out of the nucleus via the CRM1 pathway.

Integrin signaling events are dependent on the presence of

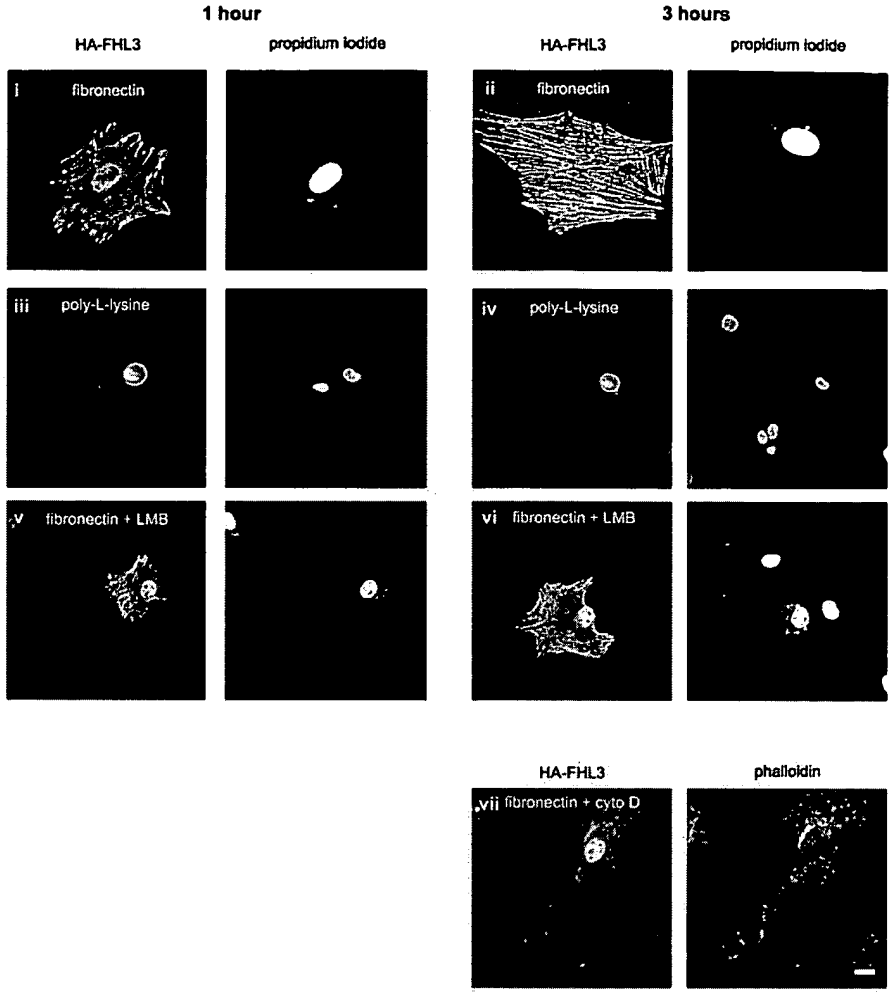

FIG. 3. **Regulation of recombinant FHL3 subcellular localization.** C2C12 cells were transiently transfected with HA-FHL3 and plated onto coverslips for analysis by confocal microscopy. Cells were plated on either fibronectin or poly-L-lysine for 1 or 3 h as indicated. In some experiments, C2C12 cells were treated with leptomycin B (2 ng/ml) (*LMB*) for the duration of the plating time (1 or 3 h) as indicated. Double immunofluorescence was performed using HA antibodies and propidium iodide. HA-FHL3 transfected cells were plated onto fibronectin-coated coverslips for 3 h prior to treatment with cytochalasin D (*cyto D*) (5 mM) for 30 min (*vii*) and co-stained with Texas Red-conjugated phalloidin. *Scale bar* equals 10 μm.

an intact cytoskeleton (54–56). The effect of cytoskeletal disruption on the localization of FHL3 in C2C12 myoblasts was investigated. Cytochalasin D caps the ends of growing actin filaments and prevents actin incorporation and inhibits integrin-mediated phosphorylation of focal adhesion proteins (57). After 3 h of plating on fibronectin, cells were treated with Me_2SO (vehicle, data not shown) or cytochalasin D for 30 min. Cells expressing HA-FHL3 treated with cytochalasin D but not Me_2SO showed marked cytoskeletal disruption as demonstrated by an absence of phalloidin staining of actin stress fibers (Fig. 3, *vii*). Under these conditions HA-FHL3 localized predominantly in the nucleus. Similar results were obtained using GFP-FHL3 (results not shown). Therefore upon cytoskeletal disruption, FHL3 accumulates in the nucleus.

Collectively these studies indicate FHL3 is expressed in the nucleus and at actin stress fibers and focal adhesions. FHL3 shuttles between the nucleus and cytoplasm, and localization is regulated by integrin activation and CRM-1-mediated nuclear export. These results suggest FHL3 may play a role in regulating integrin-mediated cytoskeletal events.

FHL3 Localizes to the Z-line of Mature Skeletal Muscle—We localized FHL3 in sections of mature mouse skeletal muscle stained with affinity-purified FHL3 antibodies (Fig. 4). In longitudinal sections at low magnification, FHL3 localized in a striated pattern along the muscle fiber (Fig. 4, *ii* and *iii*). Pre-immune sera was non-reactive with mouse skeletal muscle (Fig. 4, *i*). To characterize the location of the striations within muscle, sections were double-stained with FHL3 and α-actinin antibodies, a marker of the Z-line in skeletal muscle (58). In longitudinal sections FHL3 co-localized with α-actinin, indicating that FHL3 forms part of the Z-line (Fig. 4, *iv–vi*). Structurally the Z-line forms the boundary of each sarcomere and represents the site where α-actinin cross-links actin filaments from opposing half-sarcomeres (59). In addition, FHL3 staining extended outside the region of the Z-line, as defined by α-actinin, in the region known as the I-band. Many Z-line proteins extend beyond the Z-line into the I-band, including the barbed end of actin thin filaments (60). In transverse sections FHL3 co-localized with α-actinin at the plasma membrane and at punctate structures within the muscle fiber (Fig. 4, *vii–xii*), as shown previously for α-actinin localization (61). In transverse sections FHL3 co-localized with actin (Fig. 4, *x–xii*). Therefore, FHL3 is a component of the Z-line of skeletal muscle.

Yeast Two-hybrid Screen, FHL3 Interacts with Actin—LIM domains are protein-protein interaction motifs (3) that act as molecular scaffolds. To determine the molecular basis for FHL3 association with actin stress fibers and at the Z-line, a yeast two-hybrid screen was performed to identify FHL3-interacting proteins. We were unable to express intact FHL3 in transformed yeast cells. Therefore, the first two and half LIM domains of FHL3 were expressed in yeast cells with a library of proteins expressed as fusions with the GAL4 transcription activation domain. Several rounds of screening of a human skeletal muscle cDNA library identified a number of interacting clones, which grew on selective media, suggesting the presence of *bona fide* interactions for the first two and a half LIM domains. Sequence analysis demonstrated that one clone, a partial cDNA encoding the α-skeletal actin gene (amino acids 205–377), ACTA1, was in-frame with the GAL4 activation domain. Several controls were performed to assess the fidelity of

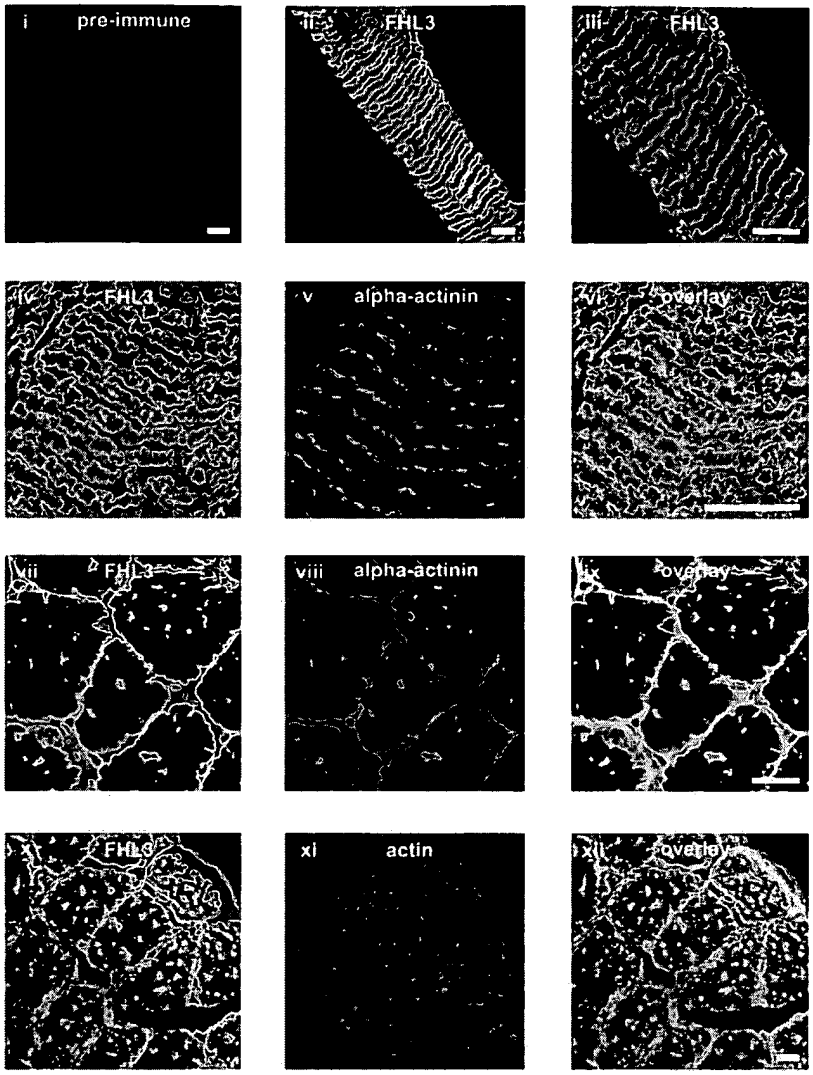

FIG. 4. **Localization of FHL3 in mouse soleus muscle.** Frozen longitudinal (*i–vi*) or transverse (*vii–xii*) sections of mouse soleus muscle were stained with pre-immune sera (*i*) or affinity-purified FHL3 anti-peptide antibodies (*ii–iv, vii,* and *x*). Double immunofluorescence was performed with affinity-purified FHL3 anti-peptide antibodies and α-actinin antibodies (*v* and *viii*) or anti-actin antibodies (*xi*) (as indicated). Samples were analyzed by confocal microscopy. Overlay images are shown in *vi, ix,* and *xii*. *Scale bars* equal 10 μM.

this interaction in yeast. Neither the bait nor ACTA1 autonomously activated the reporter genes in the yeast two-hybrid system when grown on appropriate nutrient-deficient media (minus amino acids histidine, leucine, adenine, and tryptophan with the addition of 15 mM 3-amino-1,2,4 triazole).

Identification of FHL3 Domains Mediating the Interaction with Actin in Yeast Two-hybrid Analysis and Specificity of These Interactions—To identify the region of FHL3 responsible for the interaction with actin, the activation domain plasmid containing the actin clone identified in the screen, was transformed into the yeast strain Y187. This strain was then mated with the AH109 yeast strain expressing various LIM domains encoded by FHL1, FHL2, and FHL3 in-frame with the binding domain (BD). Mated yeast were plated onto selective media, and interactions were scored as strong (+++) or weak (+). A strong interaction was demonstrated using the first two and half LIM domains of FHL3 or the second and third FHL3 LIM domains with actin (Fig. 5). Therefore, the second LIM domain may be responsible for the FHL3 interaction with actin, although maximum binding may rely on the coordination between two LIM domains. FHL3 LIM domains 3 and 4 interacted only weakly with actin. Baits expressing partial coding regions of other FHL proteins, including FHL2, FHL1, and KyoT2, were also investigated for interaction with actin. No interaction was demonstrated using any FHL1 LIM domain or KyoT2 with actin. However, the third and fourth LIM domain of FHL2 strongly interacted with actin.

FHL3 Binds Actin Both in Vitro and in Vivo—*In vitro* studies were performed to demonstrate a direct interaction between FHL3 and actin. GST or GST-FHL3 fusion proteins were bound to glutathione-Sepharose and incubated with purified rabbit skeletal muscle actin. After extensive washing, Sepharose pellets were immunoblotted with anti-actin antibodies to detect complexed actin or anti-GST antibodies to demonstrate equal loading of fusion proteins. Actin bound to GST-FHL3 but not GST (Fig. 6*A*). Immunoblot analysis using anti-GST antibodies demonstrated equal loading of GST and GST-FHL3 recombinant proteins bound to glutathione-Sepharose. Therefore FHL3 directly binds actin *in vitro*, consistent with yeast two-hybrid analysis.

Association of FHL3 and actin was demonstrated in COS-1 cells, which were co-transfected with FLAG-tagged α-skeletal actin (FLAG-actin) and HA-FHL3. Cell lysates were immunoprecipitated with anti-FLAG antibodies or non-immune antibodies, and immunoprecipitates were immunoblotted using antibodies to the FLAG or HA tags (Fig. 6*B*). HA-FHL3 was detected in FLAG but not non-immune immunoprecipitates (Fig. 6*B, lower panel*). In control experiments, to exclude nonspecific interaction mediated by the HA and FLAG tags, cells were co-transfected with HA-β-galactosidase and FLAG-actin. HA-β-galactosidase was not detected in FLAG immunoprecipitates (not shown).

To determine whether FHL3 and actin form a complex in muscle cells, C2C12 myoblast cell lysates were immunoprecipi-

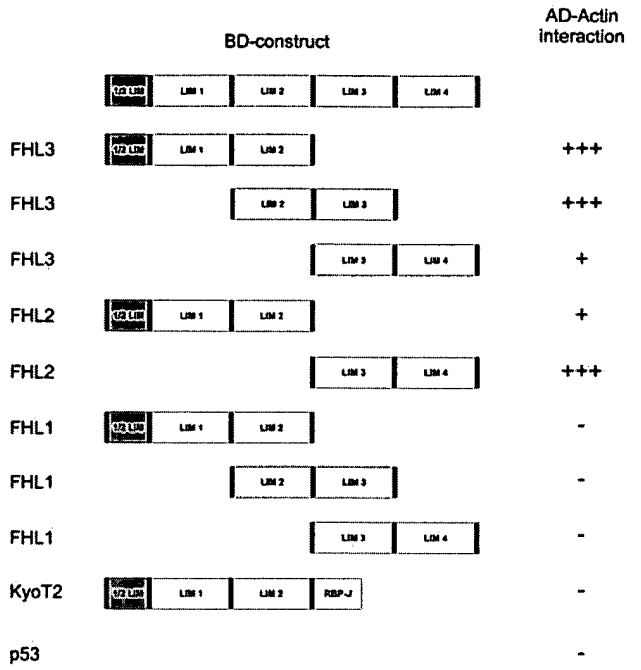

FIG. 5. **Identification and specificity of FHL3 LIM domain interaction with actin.** AH109 yeast expressing GAL4-DNA-binding domain (*BD*) fused to the indicated LIM domains of FHL3, FHL2, FHL1, KyoT2, or p53 (as a negative control) were mated with Y187 yeast expressing GAL4-activation domain (*AD*) fused to α-skeletal actin (*AD-actin*). The transformants were plated onto media lacking tryptophan, leucine, adenine, and histidine and assessed for growth and LacZ activity. A strong or weak interaction is indicated by +++ or +, respectively. No interaction is indicated by −. BD-p53 was used as a negative control and showed no interaction with AD-actin.

tated with FHL3 antibodies and immunoblotted with actin antibodies. A single polypeptide of 42 kDa, consistent with actin, was detected in FHL3 immune but not preimmune immunoprecipitations (Fig. 6*C, lower panel*). In control studies FHL3 immunoprecipitates were immunoblotted with FHL3 antibodies and demonstrated a 36-kDa polypeptide in immune but not preimmune immunoprecipitates (Fig. 6*C, upper panel*). Thus endogenous FHL3 and actin form a complex *in vivo* in C2C12 myoblasts.

As α-actinin binds actin (58), and FHL3 co-localizes with α-actinin at actin stress fibers and at the Z-line of mature skeletal muscle, we also investigated whether FHL3 could directly bind α-actinin. By using GST pull-down assays, we were unable to demonstrate purified α-actinin-bound GST-FHL3 (results not shown). In addition we were unable to demonstrate by co-immunoprecipitation of co-transfected FLAG-α-actinin and HA-FHL3 that these two species formed a complex in intact cells (results not shown).

FHL3 Promotes Spreading in C2C12 Myoblasts—To characterize the role FHL3 plays in regulating actin dynamics *in vivo*, GFP-FHL3 was overexpressed in C2C12 myoblasts, and the effect on cell spreading was determined. Cells were transfected with either GFP empty vector or GFP-FHL3 and plated onto fibronectin-coated coverslips for 15, 60, or 180 min. At each time point, the percent spread cells, defined as cells in which the surface area of the cytoplasm was at least twice the surface area of the nucleus, was determined (Fig. 7, *A* and *B*). To identify the nucleus, fixed cells were stained with propidium iodide (red staining). At 15 min both GFP-FHL3 and GFP-expressing cells adhered to the substratum but had not spread. At 60 min 50% of the GFP-FHL3-transfected cells were spread, compared with only 20% of the GFP empty vector-transfected cells. At 180 min after plating, 60% of the GFP-FHL3 cells were spread, compared with 52% of GFP empty vector cells. Therefore, particularly at the 60 min time point in early spreading, overexpression of FHL3 enhanced cell spreading following cell adhesion.

FHL3 Overexpression Disrupts the Actin Cytoskeleton in C2C12 Myoblasts—We further investigated the role of FHL3 in regulating the actin cytoskeleton in fully spread cells, including the turnover of stress fibers, or focal adhesions. In previous experiments using transient transfection of HA-FHL3, we noted a population of cells that had spread for 3 h on a fibronectin matrix, demonstrated abnormalities in actin stress fibers. This phenotype was detected only in cells expressing FHL3 at high levels. To characterize further FHL3-induced cytoskeletal rearrangement, cells were transfected with either HA-β-galactosidase as a control or HA-FHL3 and plated onto fibronectin-coated coverslips for 3 h. Cells were fixed and stained with anti-HA antibodies to detect FHL3 expression and also co-stained with phalloidin (Fig. 8*A*). HA-β-galactosidase overexpression did not influence the formation of actin stress fibers (Fig. 8*A, i* and *ii*). In contrast, HA-FHL3 expression at high levels correlated with loss of phalloidin staining of stress fibers. In cells expressing high levels of HA-FHL3, FHL3 localization appeared diffusely cytoplasmic, and no stress fibers were stained with phalloidin (Fig. 8*A, iii* and *iv*). However, F-actin was still detected in these cells, as shown by diffuse cytoplasmic phalloidin staining and staining of submembranous actin. In low HA-FHL3-expressing cells, HA-FHL3 localized to actin stress fibers, and the actin stress fibers were intact (Fig. 8*A, iii* and *iv*). To correlate the changes in actin stress fibers with focal adhesions, HA-FHL3-expressing cells were triple-labeled with antibodies to the HA tag, phalloidin staining, and anti-paxillin antibodies (Fig. 8*B*). In low HA-FHL3-expressing cells, actin stress fibers were detected, and there was little change in focal adhesion number or size (Fig. 8*B, i–iii*). However, upon high FHL3 expression, focal adhesions were both smaller in size, and decreased intensity paxillin staining was noted, associated with loss of actin stress fibers as shown by phalloidin staining (Fig. 8*B, vii–ix*). Similar studies were undertaken for HA-β-galactosidase-expressing cells and demonstrated no changes in the number or size of focal adhesions (not shown). To exclude the possibility that overexpression of FHL-3 affects cell viability, live cells expressing HA-FHL-3 or HA-β-galactosidase were co-stained with tetramethylrhodamine methyl ester perchlorate (TMRM), which is incorporated into the mitochondria of metabolically active cells, and propidium iodide which stains the nucleus of apoptotic cells. Cells expressing both low and high levels of HA-FHL3 were compared with HA-β-galactosidase-expressing cells. All transfected cells demonstrated equal intensity fluorescence of mitochondrial TMRM, and no propidium iodide staining of the nucleus was detected, indicating cell viability was not affected by high level FHL3 expression (results not shown). Collectively, these results suggest FHL3 binds actin and when expressed at high levels results in actin stress fiber disassembly.

FHL3 Inhibits the Actin Cross-linking Activity of α-Actinin—FHL3 localizes at actin stress fibers in myoblasts and at Z-lines in striated muscle, both sites of actin filament anchorage. The ability of FHL3 to bind actin both *in vitro* and *in vivo* suggests that FHL3 may regulate actin dynamics. Furthermore, significant overexpression of FHL3 results in actin stress fiber disassembly. The actin cross-linker α-actinin contributes to the maintenance and stability of actin stress fibers. To investigate further the molecular mechanisms responsible for actin stress fiber disassembly, mediated by an interaction between FHL3 and actin, actin co-sedimentation assays were performed using

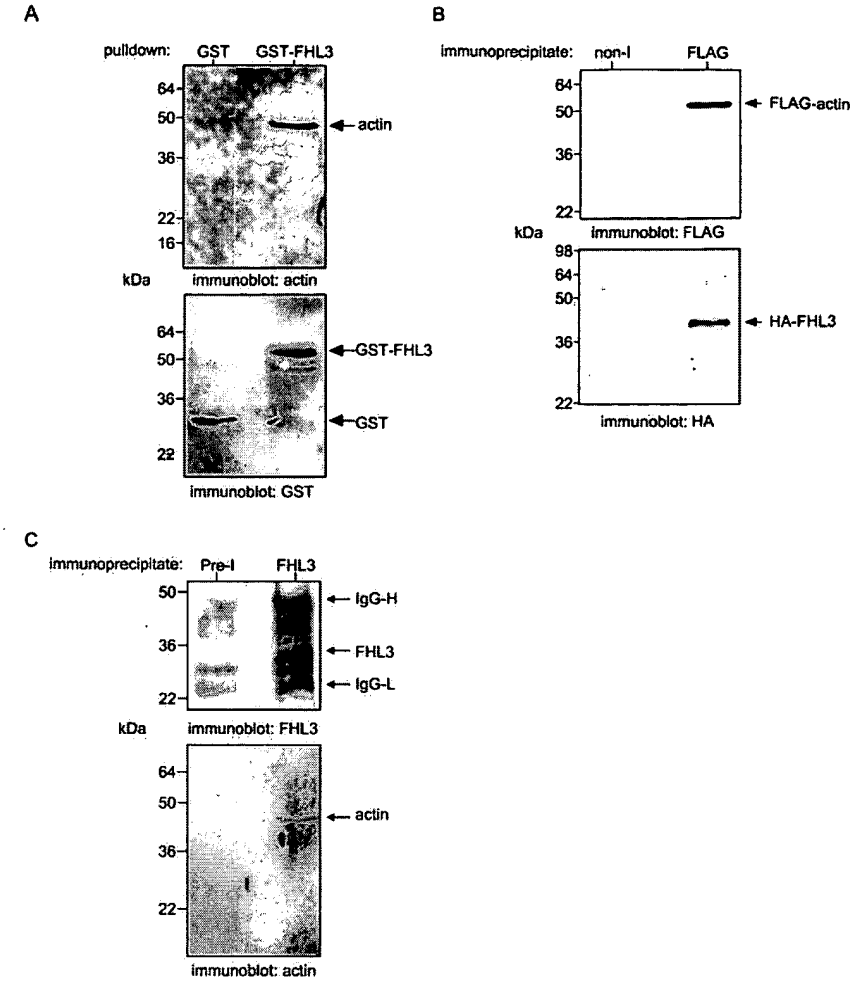

FIG. 6. **FHL3 interacts with actin *in vitro* and *in vivo*.** *A*, purified recombinant GST or GST-FHL3 fusion protein (0.5 μmol) coupled to glutathione-Sepharose was incubated with 4 μM purified muscle actin in actin binding assays as described (see "Experimental Procedures"). After extensive washing, Sepharose beads were analyzed by SDS-PAGE and immunoblotting using actin (*upper panel*) or GST (*lower panel*) antibodies. The migration of molecular mass markers are shown on the *left*. *B*, COS-1 cells were transiently transfected with HA-tagged FHL3 (*HA-FHL3*) and FLAG-tagged α-skeletal actin (*FLAG-actin*). Cell lysates were immunoprecipitated with anti-FLAG or non-immune (*non-I*) mouse monoclonal antibodies as indicated. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG (*upper panel*), or HA (*lower panel*). *C*, C2C12 cell lysates were immunoprecipitated with pre-immune sera or affinity-purified FHL3 antibodies as indicated and analyzed by SDS-PAGE and immunoblotting using FHL3 (*upper panel*) or actin antibodies (*lower panel*).

highly purified muscle actin in the presence or absence of α-actinin, which cross-links and co-sediments with actin (62). To determine whether FHL3 inhibited α-actinin-mediated actin bundling, actin filaments were incubated alone, with α-actinin, or with α-actinin in the presence or absence of GST or GST-FHL3. After incubation, bundled F-actin and its associated proteins were pelleted by centrifugation at low speed 10,000 $\times$ *g*. Pellets and supernatants were examined by SDS-PAGE and staining with Coomassie Brilliant Blue. In the absence of α-actinin the majority of actin remained in the supernatant (S) (Fig. 9*A*, *lane 1S*), and little actin was detected in the pellet (*lane 1P*). The addition of the actin-bundling protein α-actinin enhanced the amount of actin present in the pellet (Fig. 9*A*, *lane 2P*), compared with actin alone (Fig. 9*A*, *lane 1P*). In addition, α-actinin co-sedimented with actin bundles (Fig. 9*A*, *lane 2P*, see *arrow*). In the absence of α-actinin, neither GST nor GST-FHL3 had any effect on actin filament bundling and neither recombinant protein co-sedimented with bundled actin (Fig. 9*A*, *lanes 3P* and *4P*). The inclusion of GST to actin and α-actinin had no effect on α-actinin-mediated actin bundling (Fig. 9*A*, *lane 5P*), which was similar to that detected with actin and α-actinin alone. In this reaction α-actinin co-sedimented with actin and was detected with the pellet. However, the addition of GST-FHL3 resulted in significantly decreased actin bundling (Fig. 9*A*, *lane 6P*, see *arrow* for α-actinin) as shown by the recovery of actin in the pellet, which decreased to a level equivalent to that observed in the absence of α-actinin (Fig. 9*A*, *lane 1P*). In addition it was noteworthy that in the presence of GST-FHL3, α-actinin failed to co-sediment with actin (Fig. 9*A*, *lane 6P* see *arrow* for α actinin).

Densitometry was performed on the actin pellet fractions of Coomassie-stained gels of three independent low speed sedimentation experiments to quantitate the effects of GST-FHL3 on actin sedimentation (Fig. 9*B*). A two and a half-fold increase in actin recovery in the pellet was observed in the presence of α-actinin compared with actin alone (Fig. 9*B*, *lane 2 versus lane 1*). The addition of GST did not affect this result (Fig. 9*B*, *lane 5*). However, GST-FHL3 completely inhibited the sedimentation of actin by α-actinin (Fig. 9*B*, *lane 6*). These results indicate that FHL3 either induces F-actin depolymerization or inhibits α-actinin-induced actin bundling.

To confirm further the effect of GST-FHL3 on actin bundling, the supernatants and pellets of these low speed sedimentation experiments were analyzed by immunoblot with actin antibodies (Fig. 9*C*). The presence of α-actinin enhanced the recovery of actin bundles in the pellet (Fig. 9*C*, *lane 2*) compared with that observed in the absence of α-actinin (Fig. 9*C*, *lane 1*). The presence of GST-FHL3 greatly reduced the recovery of actin in the pellet in both the absence and more notably in the presence of α-actinin (Fig. 9*C*, *lanes 4* and *6*, respectively), although in control studies GST alone had no effect on the amount of actin pelleted in the presence of α-actinin (Fig. 9*C*, *lane 5*). Immunoblots of the supernatant of these sedimentation assays confirmed equal loading of actin in the reactions (Fig. 9*C*, *lower panel*).

In order to analyze directly the effects of FHL3 on actin filament bundling, we performed electron microscopy on negatively stained actin filaments (62, 63). Single actin filaments were observed across the grid when polymerized actin was

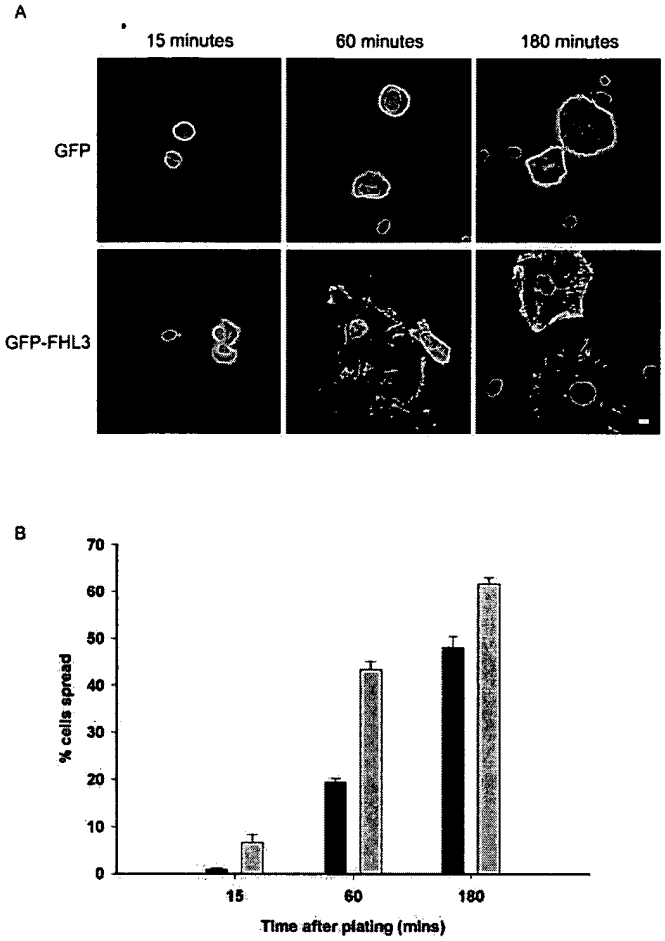

FIG. 7. **GFP-FHL3 increases the rate C2C12 myoblasts cell spreading.** *A*, C2C12 cells were transfected with GFP or GFP-FHL3 (as indicated) and plated onto fibronectin-coated coverslips for 15, 60, or 180 min (as indicated) prior to cell fixation. Cells were co-stained with propidium iodide (*red* staining) to visualize the nucleus and analyzed by confocal microscopy. *Scale bar* equals 10 μm. *B*, cells were counted and scored for spreading. *Black bars* represent GFP-transfected cells and *gray bars* GFP-FHL3-transfected cells. Cells were counted as spread if the cytoplasmic surface area was more than twice that of the surface area of the nucleus. Results are presented as the percentage of transfected cells counted that appeared spread. 100 cells per coverslip were counted in three separate experiments. *Error bars* indicate S.E.

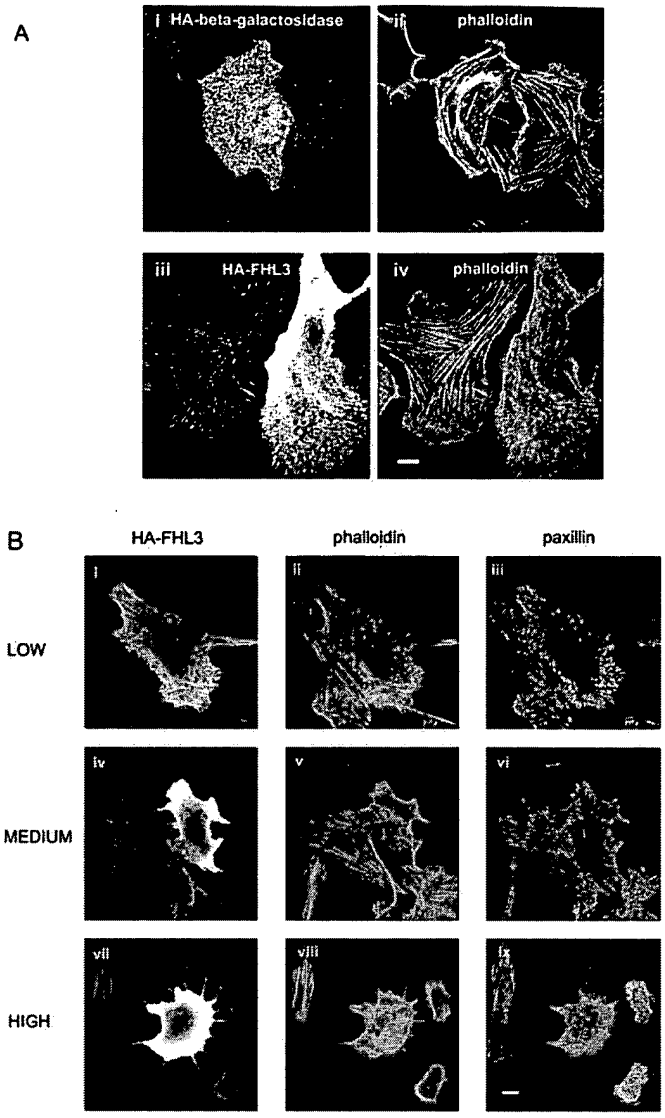

FIG. 8. **High level FHL3 overexpression disassembles actin stress fibers.** *A*, C2C12 cells were transfected with HA-β-galactosidase, as a control (*i* and *ii*), or HA-FHL3 (*iii* and *iv*), and plated onto fibronectin-coated coverslips for 3 h prior to fixing. Cells were stained with HA antibodies (*left column*) and phalloidin (*right column*). *Scale bars* equals 10 μm. *B*, C2C12 cells were transfected with HA-FHL3 and plated onto fibronectin-coated coverslips for 3 h prior to fixing. Cells were triple-labeled for anti-HA (*left panel*), phalloidin (*central panel*), or anti-paxillin antibodies (*right panel*). *Scale bar* equals 10 μm. The level of HA-FHL3 expression, as determined by the intensity of anti-HA staining, is indicated on the *left* as low, medium, or high.

incubated alone (Fig. 9*D, i*). In the presence of α-actinin, actin filaments formed thick bundles (Fig. 9*D. ii*). The inclusion of GST had no effect on the ability of α-actinin to bundle actin (Fig. 9*D, iii*). However, in the presence of GST-FHL3 with α-actinin and actin, no actin bundles were detected across the grid (Fig. 9*D, iv*). There was no apparent decrease in the number or appearance of the actin filaments. These results are consistent with the co-sedimentation assays and suggest FHL3 inhibits α-actinin bundling of actin filaments, rather than causing depolymerization of actin filaments. The observation that overexpression of FHL3 results in decreased actin stress fibers suggests that by inhibiting α-actinin-mediated actin cross-linking, FHL3 may cause actin stress fibers to become less stable and disassemble.

FHL3 Expression Increases in C2C12 Myoblasts Migrating into a Wound Edge—We have demonstrated that overexpression of FHL3 induces actin stress fiber disassembly via inhibition of α-actinin-mediated actin cross-linking. We therefore investigated whether increased FHL3 expression could be demonstrated *in vivo* in cells rapidly restructuring actin stress fibers, such as migrating cells. The level of expression of endogenous FHL3 in C2C12 myoblasts migrating into a wound was determined. Cells were plated onto fibronectin-coated coverslips, grown to confluence for 12 h, and then the cell monolayer wounded using a plastic pipette (36). The wound was allowed to close for 24 h before cells were stained with FHL3 antibodies (Fig. 10). By using a computer-generated glow-over scale, the expression levels of FHL3 were compared in non-migrating confluent cells, distant from the wound edge, to cells migrating into the wound. The highest levels of FHL3 expression appear *blue*, intermediate levels as *white*, and lower level expression as *red*. In the confluent non-migratory cells, FHL3 staining was predominantly red, indicating lower level expression. Toward the wound edge, the level of FHL3 expression increased as indicated by predominantly white-stained cells. The most highly motile single cells migrating into the wound

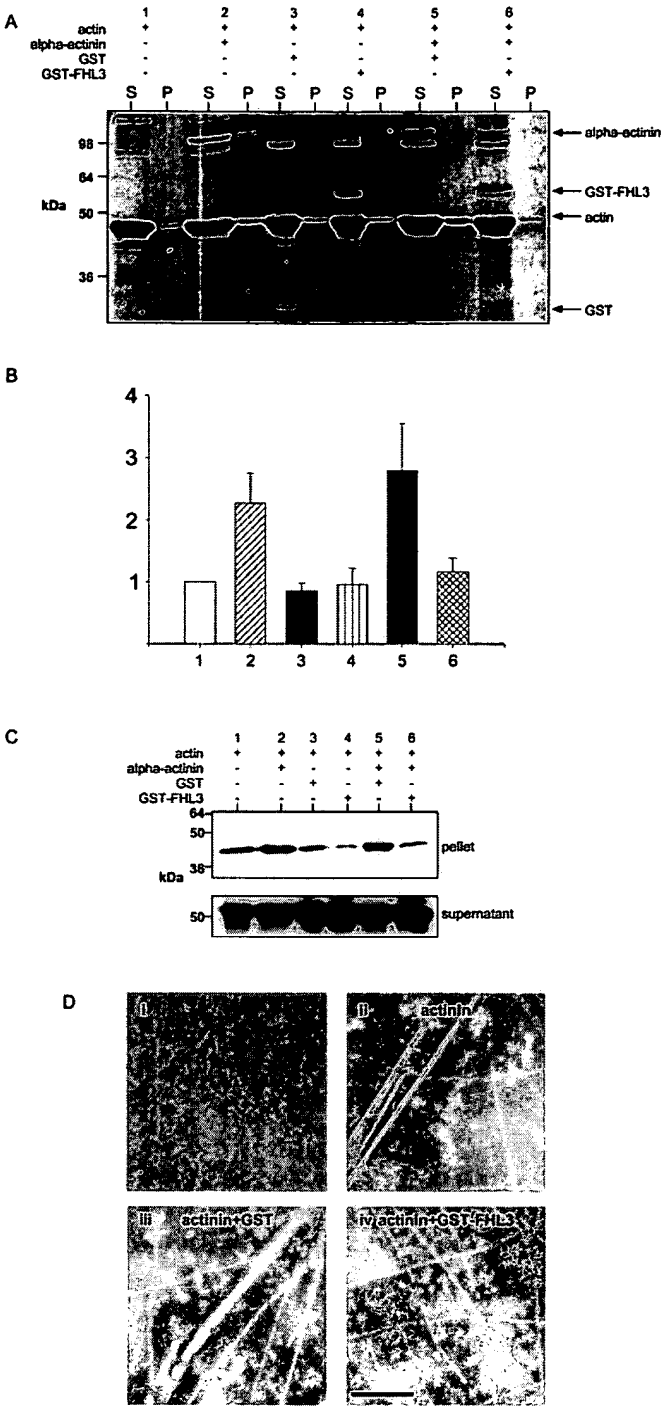

FIG. 9. **FHL3 inhibits the actin cross-linking activity of α-actinin.** *A*, F-actin (4 μM) was incubated for 2 h with or without 250 nM purified α-actinin and in the presence or absence of GST (250 nM) or GST-FHL3 (250 nM) as indicated. Bundled actin and associated proteins were pelleted by centrifugation at 10,000 × *g* for 10 min, and the supernatants (*S*) and pellets (*P*) were analyzed by SDS-PAGE and Coomassie staining. *Arrows* on the *right* indicate the migration of α-actinin, GST-FHL3, actin, or GST. The migration of molecular mass markers is shown on the *left*. *B*, densitometry analysis of the Coomassie-stained actin in pellet fractions from three low speed sedimentation experiments as performed in *A*. Data are presented as a ratio of actin detected in the pellet normalized to that recovered in the absence of α-actinin (*B, bar 1*). *Bar numbers* correspond to the corresponding pellet numbers in *A*. *Error bars* indicate S.E. *C*, F-actin was incubated with recombinant proteins as described in *A*, and analysis of actin in the supernatant (*lower panel*) and pellet (*upper panel*) was performed by SDS-PAGE and immunoblotted with actin antibodies. The migration of molecular mass markers is shown on the *left*. *D*, electron microscopy of negatively stained actin filaments was performed with the following combinations of purified proteins: (*i*) 2 μM actin; (*ii*) 2 μM actin and 500 nM α-actinin; (*iii*) 2 μM actin, 500 nM α-actinin, and 500 nM GST; and (*iv*) 2 μM actin, 500 nM α-actinin, and 500 nM GST-FHL3.

primarily had blue staining, indicating the highest level of FHL3 expression. In control studies no changes were noted in the intensity of phalloidin staining of actin in migrating cells (Fig. 10). These results indicate that FHL3 is up-regulated in migrating C2C12 cells at the wound edge and, together with our earlier observations, suggest that increased FHL3 expression may contribute to cytoskeletal rearrangement during myoblast migration.

DISCUSSION

The results of this study have shown FHL3 is an actin-binding protein that inhibits α-actinin-mediated bundling of actin fibers. By using multiple approaches we have provided evidence that FHL3 regulates the actin cytoskeleton. FHL3 binds actin both *in vitro* and *in vivo* as shown, respectively, by direct binding assays using recombinant GST-FHL3 and purified actin, and by co-immunoprecipitation of endogenous and recombinant FHL3 with actin from mammalian cells. In addition, FHL3 co-localizes with actin and α-actinin at stress fibers in myoblasts and at the Z-line of mature striated muscle. In myoblasts the cycling of FHL3 between the nucleus and the cytoskeleton is regulated by integrin activation. FHL3 inhibits α-actinin-mediated cross-linking of actin *in vitro*. In intact cells, FHL3 regulates cell spreading and actin stress fiber disassembly. We have provided evidence that FHL3 expression is increased in migrating myoblasts. Collectively, the studies reported here demonstrate FHL3 plays a significant role in regulating the actin cytoskeleton in myoblasts.

FHL3 localization in C2C12 myoblasts to stress fibers and focal adhesions was dependent on integrin activation. Although recombinant FHL3 has been reported previously to localize to focal adhesions (22), the localization of this LIM protein to actin stress fibers has not been described. Furthermore, we have also shown that integrin activation stimulates nuclear exit of FHL3 and re-localization at actin stress fibers. Although FHL3 lacks a classical lysine-rich nuclear export sequence (52, 53), FHL3 nuclear export was sensitive to leptomycin B, a specific inhibitor of CRM-1-dependent nuclear export (51). Actin contains two leptomycin B-sensitive lysine-rich nuclear export sequences (64), and thus actin binding to FHL3 may facilitate FHL3 nuclear export. Another FHL family member FHL1 shows a similar integrin-dependent nuclear to cytoplasmic re-localization to stress fibers and focal adhesions (16). Furthermore FHL1, like FHL3, promotes cell spreading. However, the molecular mechanisms mediating FHL1 regulation of cell spreading are unknown. It is unlikely to be via the same mechanism as FHL3, as we were unable to demonstrate that FHL1 interacts with actin in yeast two-hybrid assays. In addition, we have not observed FHL1-induced disruption of actin stress fibers, when this LIM protein is overexpressed in C2C12 myoblasts (results not shown), suggesting distinct roles for FHL1 and FHL3, both of which are highly expressed in striated muscle (4). Another FHL protein, FHL2, binds both the integrin receptor and β-catenin and also localizes to focal adhesions (21, 27, 28). We have shown in this study that FHL2, like FHL3, binds actin in yeast two-hybrid studies and are currently investigating whether FHL2 forms a functionally significant complex with actin in myoblasts. Recent studies (27) have shown overexpression of FHL2 in C2C12 cells accelerates myogenic differentiation and accelerates myotube formation. However, in these stud-

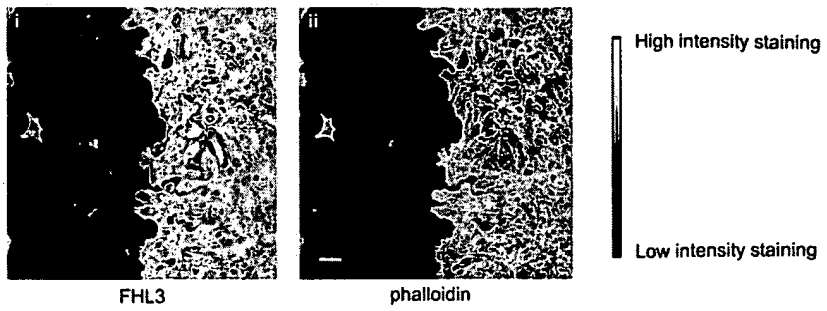

FIG. 10. **Expression and subcellular localization of FHL3 in migrating C2C12 myoblasts.** A confluent monolayer of C2C12 myoblasts, plated onto fibronectin-coated coverslips, was wounded and fixed 24 h after wounding. Cells were stained with FHL3 antibodies (*left panel*) or phalloidin staining (*right panel*) and confocal images were taken using the glow-over function to grade the intensity of staining. Low level staining intensity is indicated by *black-red* staining and high level by *white-blue* staining as indicated in the color scale on the *right*. *Scale bar* equals 50 μm.

ies FHL2 localization to and regulation of actin stress fibers was not described.

In differentiated muscle both FHL2 and, as reported here, FHL3 localize to the Z-line of cardiac myocytes (26, 65) and skeletal muscle, respectively. FHL2 is predominantly expressed in cardiac muscle and FHL3 in skeletal muscle (6, 17), suggesting these LIM proteins may serve functionally nonredundant roles. We have localized FHL3 in mature muscle to the Z-line, a site where overlapping anti-parallel actin filaments are cross-linked from adjacent sarcomeres. Several proteins, such as myopodin, a recently identified actin bundling protein, and the LIM domain-containing proteins MLP and ALP, promote α-actinin-induced actin bundling (62, 66, 67). Specific changes in physical forces and stress may require constant remodeling of α-actinin-induced actin bundling at the Z-line (66). Therefore, it is not entirely surprising that there should be proteins, such as FHL3, that have the potential to negatively regulate actin bundling at this site, in addition to proteins that promote actin bundling.

Until recently, very little was known of the biological function of the FHL family of proteins. Emerging evidence has shown recently (10, 18, 19) several FHL family members including FHL3, FHL2, and ACT can serve as co-activators for transcription factors including CREM/CREB and the androgen receptor. In addition, both FHL2 and FHL3 transcriptional co-factor activities are regulated by activation of the Rho signaling pathway, with FHL3 demonstrating the strongest activity (31). It has not been directly shown that Rho activation leads to nuclear accumulation of FHL3, rather constitutively active Rho stimulates FHL3 transcriptional co-factor activity, suggesting FHL3 must be in the nucleus for this to occur (19). We have demonstrated FHL3 is present in the nucleus 1 h after plating cells on fibronectin, but by 3 h has exited the nucleus in the majority of cells. Previous studies have demonstrated RhoA activity is regulated in a triphasic manner by fibronectin. Rho activity is initially inhibited by fibronectin via c-Src-dependent activation of 190RhoGAP, which facilitates cell spreading (68). In the second phase, 45–90 min after fibronectin stimulation, RhoA is activated correlating with stress fiber and focal adhesion formation. However, by 120 min RhoA activity is reduced to basal levels, which is proposed to be required for membrane protrusion and cell elongation. The exit of FHL3 from the nucleus of cells plated for 3 h on fibronectin correlates with the time frame in which Rho activity is largely diminished. As yet, the degree of Rho activation required to stimulate nuclear accumulation of FHL3 is unknown. Also, it is likely there are factors in addition to Rho that influence the nuclear accumulation and transcriptional co-factor activity of FHL3 in C2C12 cells. However, it is noteworthy cytochalasin D causes an increase in Rho activation (69), and under these circumstances we have demonstrated significant FHL3 nuclear accumulation.

LIM proteins have demonstrated roles in regulating cytoskeletal integrity. The CRP family (CRP1, CRP2, and CRP3/MLP) localizes to actin stress fibers and focal adhesions and directly interacts with α-actinin and zyxin (67), whereas MLP binds β-spectrin via its C-terminal LIM domain (33). CRPs stabilize actin-rich structures in muscle. Gene-targeted deletion of MLP leads to cyto-architectural disorganization of cardiac and skeletal muscle (70). Recently two novel LIM proteins from *Dictyostelium,* LIM C and LIM D, have been identified that directly bind actin. Gene-targeted mutants of these novel LIM-only proteins demonstrate significantly impaired growth under stress conditions, suggesting these proteins regulate the maintenance of cortical strength (71). The majority of LIM domain proteins interact with actin indirectly via interaction with zyxin or α-actinin or via specific actin-binding domains, independent of their LIM domains (67, 72). It is noteworthy that LIM C and D, and as shown in this report FHL3, are the first LIM-only proteins shown to directly bind actin via their LIM domains. In addition, results of the yeast two-hybrid screening suggest that actin binding is not a function of all FHL family members as only FHL3 and FHL2, but not FHL1 and KytoT2, demonstrated this activity. Furthermore, no other LIM protein to our knowledge has been shown previously to inhibit actin bundling by α-actinin or by any other actin bundling/cross-linking protein.

α-Actinin facilitates the stabilization of stress fibers and focal adhesions against mechanical perturbations (73). In striated muscle α-actinin is localized to the Z-disc (58) where it co-localizes with FHL3. The co-factors that regulate α-actinin binding to F-actin are not well described and have yet to be fully identified. α-Actinin has the ability to cross-link actin filaments in any orientation, thereby making α-actinin unlikely by itself to mediate the formation of bundled actin filaments in a directed polar orientation. It has been proposed that the polarity of actin-containing structures may be mediated by components of the cytoskeleton that limit access of α-actinin to actin (74). In this regard we have demonstrated that FHL3 can inhibit α-actinin actin bundling activity. As FHL3 also binds actin, this LIM protein may compete with α-actinin for actin binding. FHL3, unlike other LIM proteins such as MLP and zyxin, does not directly bind α-actinin (67, 72). FHL3 binding to actin may prevent α-actinin, once bound to actin, from cross-linking actin filaments. Furthermore, it is likely FHL3 binds other signaling or structural proteins via its four and a half LIM domains that may contribute to the regulation of α-actinin cross-linking activity *in vivo*.

We have shown in this study that FHL3 promotes cell spreading. Following cell adhesion and integrin activation, cells rearrange their cytoskeleton and spread by increasing their contact with the extracellular matrix and through the formation of stress fibers and focal adhesions (49, 75, 76). However, in addition we have also shown when FHL3 is expressed at high levels in the spread cell, actin stress fibers

disassemble consistent with de-adhesion, an intermediate state of adhesion characterized by restructuring of actin stress fibers, favoring cell motility (77, 78). We have shown a marked increase in the expression of FHL3 in myoblasts migrating into an artificial wound. This is consistent with the contention that in migrating cells high levels of FHL3 expression may mediate restructuring of stress fibers and thus facilitate cell migration. However, we were unable to demonstrate that C2C12 cells transiently expressing GFP-FHL3 showed enhanced cell migration using Boyden chamber migration assays (results not shown). By using a similar experimental approach, we have recently shown FHL1 enhances cell migration (16). Our inability to demonstrate that FHL3 regulates cell migration may relate to the variable expression levels achieved in the mixed population of transiently transfected C2C12 cells. It is of interest that modulation of α-actinin levels increases the motility and the tumorigenic properties of cells. For example, decreased α-actinin levels may contribute to malignant transformation in 3T3 cells (79, 80). In this context it is noteworthy that although FHL3 expression appears to be limited to striated muscle, examination of various malignant cell lines has shown increased FHL3 expression in melanoma and leukemia cell lines (81). Thus increased FHL3 expression may contribute to de-adhesion and the increased motility of these malignant cells. Upon cell migration, FHL3 levels are increased, which may facilitate disassembly of actin stress fibers, via inhibition of α-actinin bundling of actin.

Acknowledgments—Confocal images and electron micrographs were obtained using the facilities of Monash Micro-imaging. We thank Dr. Ian Harper for assistance in using the Confocal Facility and Gunta Jaudzems for assistance with the electron microscope. Siew Khim Hoe provided invaluable technical advice with negative staining of actin filaments.

REFERENCES

1. Dawid, I. B., Toyama, R., and Taira, M. (1995) *C. R. Acad. Sci. III (Paris)* **318,** 295–306
2. Bach, I. (2000) *Mech. Dev.* **91,** 5–17
3. Dawid, I. B., Breen, J. J., and Toyama, R. (1998) *Trends Genet.* **14,** 156–162
4. Morgan, M. J., and Madgwick, A. J. (1996) *Biochem. Biophys. Res. Commun.* **225,** 632–638
5. Genini, M., Schwalbe, P., Scholl, F. A., Remppis, A., Mattei, M. G., and Schafer, B. W. (1997) *DNA Cell Biol.* **16,** 433–442
6. Lee, S. M., Tsui, S. K., Chan, K. K., Kotaka, M., Li, H. Y., Chim, S. S., Waye, M. M., Fung, K. P., and Lee, C. Y. (1998) *Somatic Cell Mol. Genet.* **24,** 197–202
7. Lee, S. M., Tsui, S. K., Chan, K. K., Garcia-Barcelo, M., Waye, M. M., Fung, K. P., Liew, C. C., and Lee, C. Y. (1998) *Gene (Amst.)* **216,** 163–170
8. Taniguchi, Y., Furukawa, T., Tun, T., Han, H., and Honjo, T. (1998) *Mol. Cell. Biol.* **18,** 644–654
9. Brown, S., McGrath, M. J., Ooms, L. M., Gurung, R., Maimone, M. M., and Mitchell, C. A. (1999) *J. Biol. Chem.* **274,** 27083–27091
10. Fimia, G. M., De Cesare, D., and Sassone-Corsi, P. (1999) *Nature* **398,** 165–169
11. Morgan, M. J., and Madgwick, A. J. (1999) *Biochem. Biophys. Res. Commun.* **255,** 251–255
12. Chu, P. H., Ruiz-Lozano, P., Zhou, Q., Cai, C., and Chen, J. (2000) *Mech. Dev.* **95,** 259–265
13. Yang, J., Moravec, C. S., Sussman, M. A., DiPaola, N. R., Fu, D., Hawthorn, L., Mitchell, C. A., Young, J. B., Francis, G. S., McCarthy, P. M., and Bond, M. (2000) *Circulation* **102,** 3046–3052
14. Lim, D. S., Roberts, R., and Marian, A. J. (2001) *J. Am. Coll. Cardiol.* **38,** 1175–1180
15. Loughna, P. T., Mason, P., Bayol, S., and Brownson, C. (2000) *Mol. Cell. Biol. Res. Commun.* **3,** 136–140
16. Robinson, P. A., Brown, S., McGrath, M. J., Coghill, I. D., Gurung, R., and Mitchell, C. A. (2003) *Am. J. Physiol.* **284,** c681–695
17. Chan, K. K., Tsui, S. K., Lee, S. M., Luk, S. C., Liew, C. C., Fung, K. P., Waye, M. M., and Lee, C. Y. (1998) *Gene (Amst.)* **210,** 345–350
18. Fimia, G. M., De Cesare, D., and Sassone-Corsi, P. (2000) *Mol. Cell. Biol.* **20,** 8613–8622
19. Muller, J. M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., Breyer, T., Holubarsch, C., Buettner, R., and Schule, R. (2000) *EMBO J.* **19,** 359–369
20. Tanahashi, H., and Tabira, T. (2000) *Hum. Mol. Genet.* **9,** 2281–2289
21. Wixler, V., Geerts, D., Laplantine, E., Westhoff, D., Smyth, N., Aumailley, M., Sonnenberg, A., and Paulsson, M. (2000) *J. Biol. Chem.* **275,** 33669–33678
22. Li, H. Y., Ng, E. K., Lee, S. M., Kotaka, M., Tsui, S. K., Lee, C. Y., Fung, K. P., and Waye, M. M. (2001) *J. Cell. Biochem.* **80,** 293–303
23. Amaar, Y. G., Thompson, G. R., Linkhart, T. A., Chen, S. T., Baylink, D. J., and Mohan, S. (2002) *J. Biol. Chem.* **277,** 12053–12060
24. Du, X., Hublitz, P., Gunther, T., Wilhelm, D., Englert, C., and Schule, R. (2002) *Biochim. Biophys. Acta* **1577,** 93–101
25. McLoughlin, P., Ehler, E., Carlile, G., Licht, J. D., and Schafer, B. W. (2002) *J. Biol. Chem.* **277,** 37045–37053
26. Lange, S., Auerbach, D., McLoughlin, P., Perriard, E., Schafer, B. W., Perriard, J. C., and Ehler, E. (2002) *J. Cell Sci.* **115,** 4925–4936
27. Martin, B., Schneider, R., Janetzky, S., Waibler, Z., Pandur, P., Kuhl, M., Behrens, J., von der Mark, K., Starzinski-Powitz, A., and Wixler, V. (2002) *J. Cell Biol.* **159,** 113–122
28. Wei, Y., Renard, C. A., Labalette, C., Wu, Y., Levy, L., Neuveut, C., Prieur, X., Flajolet, M., Prigent, S., and Buendia, M. A. (2002) *J. Biol. Chem.* **277,** 5188–5194
29. Kong, Y., Shelton, J. M., Rothermel, B., Li, X., Richardson, J. A., Bassel-Duby, R., and Williams, R. S. (2001) *Circulation* **103,** 2731–2738
30. Chu, P. H., Bardwell, W. M., Gu, Y., Ross, J., Jr., and Chen, J. (2000) *Mol. Cell. Biol.* **20,** 7460–7462
31. Muller, J. M., Metzger, E., Greschik, H., Bosserhoff, A. K., Mercep, L., Buettner, R., and Schule, R. (2002) *EMBO J.* **21,** 736–748
32. Chrzanowska-Wodnicka, M., and Burridge, K. (1996) *J. Cell Biol.* **133,** 1403–1415
33. Flick, M. J., and Konieczny, S. F. (2000) *J. Cell Sci.* **113,** 1553–1564
34. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1991) *Current Protocols in Molecular Biology*, p. 13.11.1, John Wiley & Sons, Inc., New York
35. James, M. F., Manchanda, N., Gonzalez-Agosti, C., Hartwig, J. H., and Ramesh, V. (2001) *Biochem. J.* **356,** 377–386
36. Honda, K., Yamada, T., Endo, R., Ino, Y., Gotoh, M., Tsuda, H., Yamada, Y., Chiba, H., and Hirohashi, S. (1998) *J. Cell Biol.* **140,** 1383–1393
37. Hynes, R. O. (1992) *Cell* **69,** 11–25
38. Gullberg, D., Velling, T., Lohikangas, L., and Tiger, C. F. (1998) *Front. Biosci.* **3,** D1039–D1050
39. Salgia, R., Li, J. L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Sobhany, E. S., Sun, Y., Pisick, E., Hallek, M., Ernst, T., Tantravahi, R., Chen, L. B., and Griffin, J. D. (1995) *J. Biol. Chem.* **270,** 5039–5047
40. Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., Wang, J. Y., and Hope, T. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7457–7462
41. Aplin, A. E., Stewart, S. A., Assoian, R. K., and Juliano, R. L. (2001) *J. Cell Biol.* **153,** 273–282
42. Aplin, A. E., Hogan, B. P., Tomeu, J., and Juliano, R. L. (2002) *J. Cell Sci.* **115,** 2781–2790
43. Hirsch, E., Barberis, L., Brancaccio, M., Azzolino, O., Xu, D., Kyriakis, J. M., Silengo, L., Giancotti, F. G., Tarone, G., Fassler, R., and Altruda, F. (2002) *J. Cell Biol.* **157,** 481–492
44. Nix, D. A., and Beckerle, M. C. (1997) *J. Cell Biol.* **138,** 1139–1147
45. Nix, D. A., Fradelizi, J., Bockholt, S., Menichi, B., Louvard, D., Friederich, E., and Beckerle, M. C. (2001) *J. Biol. Chem.* **276,** 34759–34767
46. Woods, A. J., Roberts, M. S., Choudhary, J., Barry, S. T., Mazaki, Y., Sabe, H., Morley, S. J., Critchley, D. R., and Norman, J. C. (2002) *J. Biol. Chem.* **277,** 6428–6437
47. Akiyama, S. K. (1996) *Hum. Cell* **9,** 181–186
48. Nojima, Y., Morino, N., Mimura, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., Yazaki, Y., and Hirai, H. (1995) *J. Biol. Chem.* **270,** 15398–15402
49. Disatnik, M. H., and Rando, T. A. (1999) *J. Biol. Chem.* **274,** 32486–32492
50. Bershadsky, A., Chausovsky, A., Becker, E., Lyubimova, A., and Geiger, B. (1996) *Curr. Biol.* **6,** 1279–1289
51. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998) *Exp. Cell Res.* **242,** 540–547
52. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* **90,** 1051–1060
53. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) *Nature* **390,** 308–311
54. Wang, Y. G., Samarel, A. M., and Lipsius, S. L. (2000) *J. Physiol. (Lond.)* **527,** 3–9
55. Wang, Y. G., Samarel, A. M., and Lipsius, S. L. (2000) *J. Physiol. (Lond.)* **526,** 57–68
56. Canobbio, I., Lova, P., Sinigaglia, F., Balduini, C., and Torti, M. (2002) *Thromb. Haemostasis* **87,** 509–517
57. Goddette, D. W., and Frieden, C. (1986) *J. Biol. Chem.* **261,** 15974–15980
58. Jockusch, H., and Jockusch, B. M. (1980) *Dev. Biol.* **75,** 231–238
59. Tskhovrebova, L. A. (1991) *J. Muscle Res. Cell Motil.* **12,** 425–438
60. Littlefield, R., and Fowler, V. M. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 487–525
61. Nakata, T., Nishina, Y., and Yorifuji, H. (2001) *Biochem. Biophys. Res. Commun.* **286,** 156–163
62. Pashmforoush, M., Pomies, P., Peterson, K. L., Kubalak, S., Ross, J., Jr., Hefti, A., Aebi, U., Beckerle, M. C., and Chien, K. R. (2001) *Nat. Med.* **7,** 591–597
63. Sasaki, Y., Hayashi, K., Shirao, T., Ishikawa, R., and Kohama, K. (1996) *J. Neurochem.* **66,** 980–988
64. Wada, A., Fukuda, M., Mishima, M., and Nishida, E. (1998) *EMBO J.* **17,** 1635–1641
65. Scholl, F. A., McLoughlin, P., Ehler, E., de Giovanni, C., and Schafer, B. W. (2000) *J. Cell Biol.* **151,** 495–506
66. Faulkner, G., Lanfranchi, G., and Valle, G. (2001) *IUBMB Life* **51,** 275–282
67. Louis, H. A., Pino, J. D., Schmeichel, K. L., Pomies, P., and Beckerle, M. C. (1997) *J. Biol. Chem.* **272,** 27484–27491
68. Arthur, W. T., and Burridge, K. (2001) *Mol. Biol. Cell* **12,** 2711–2720
69. Ren, X. D., Kiosses, W. B., and Schwartz, M. A. (1999) *EMBO J.* **18,** 578–585
70. Arber, S., Hunter, J. J., Ross, J., Jr., Hongo, M., Sansig, G., Borg, J., Perriard, J. C., Chien, K. R., and Caroni, P. (1997) *Cell* **88,** 393–403
71. Khurana, T., Khurana, B., and Noegel, A. A. (2002) *Protoplasma* **219,** 1–12

72. Crawford, A. W., Michelsen, J. W., and Beckerle, M. C. (1992) *J. Cell Biol.* **116,** 1381–1393
73. Xu, J., Tseng, Y., and Wirtz, D. (2000) *J. Biol. Chem.* **275,** 35886–35892
74. Taylor, K. A., Taylor, D. W., and Schachat, F. (2000) *J. Cell Biol.* **149,** 635–646
75. Reddy, K. B., Bialkowska, K., and Fox, J. E. (2001) *J. Biol. Chem.* **276,** 28300–28308
76. Tanaka, K., and Itoh, K. (1998) *J. Struct. Biol.* **124,** 13–41
77. Liu, L., Schwartz, B. R., Lin, N., Winn, R. K., and Harlan, J. M. (2002) *J. Immunol.* **169,** 2330–2336
78. Greenwood, J. A., and Murphy-Ullrich, J. E. (1998) *Microsc. Res. Tech.* **43,** 420–432
79. Gluck, U., and Ben-Ze'ev, A. (1994) *J. Cell Sci.* **107,** 1773–1782
80. Gluck, U., Kwiatkowski, D. J., and Ben-Ze'ev, A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 383–387
81. Morgan, M. J., and Whawell, S. A. (2000) *Biochem. Biophys. Res. Commun.* **273,** 776–783

SeqServer®
biology in silico

BLAST2 Search Results

EXHIBIT A
Docket No.: PF-0585 USN
USSN: 09/786,135

Sequences | Help

Retrieval | BLAST2 | FASTA | ClustalW | GCG Assembly | Phrap | Translation

BLAST2 Manual

 SeqServer Version 4.6 Jan 2002

Program: blastp
Sequence ID(s):
☐ 1925017CD1 vs. genpept137

NCBI-BLASTP 2.0.10 [Aug-26-1999]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= 1925017CD1
(280 letters)

Database: genpept137
1,534,369 sequences; 474,463,515 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
☑ g30584447 Homo sapiens four and a half LIM domains 3 [synthet	641	0.0
☑ g30582943 four and a half LIM domains 3 [Homo sapiens]	641	0.0
☑ g15079780 Unknown (protein for MGC:19547) [Homo sapiens]	641	0.0
☑ g12655007 Unknown (protein for MGC:8696) [Homo sapiens]	641	0.0
☑ g4894847 LIM protein [Mus musculus]	637	0.0
☑ g7381058 LIM-only protein FHL3 [Homo sapiens]	633	e-180
☑ g30526305 four and a half LIM domains 3 [Sus scrofa]	624	e-177
☑ g5825393 four and half LIM domain protein 3 [Mus musculus]	617	e-175
☑ g4416530 skeletal muscle LIM-protein FHL3 [Homo sapiens]	615	e-175
☑ g8131974 LIM protein FHL3 [Mus musculus]	487	e-136

>g30584447 Homo sapiens four and a half LIM domains 3 [synthetic construct]
Length = 281

Score = 641 bits (1635), Expect = 0.0
Identities = 280/280 (100%), Positives = 280/280 (100%)

```
Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
```

>g30582943 four and a half LIM domains 3 [Homo sapiens]
Length = 280

Score = 641 bits (1635), Expect = 0.0
Identities = 280/280 (100%), Positives = 280/280 (100%)

```
Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
```

>g15079780 Unknown (protein for MGC:19547) [Homo sapiens]
Length = 280

Score = 641 bits (1635), Expect = 0.0
Identities = 280/280 (100%), Positives = 280/280 (100%)

```
Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
```

```
Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280


>g12655007 Unknown (protein for MGC:8696) [Homo sapiens]
           Length = 280

 Score =  641 bits (1635), Expect = 0.0
 Identities = 280/280 (100%), Positives = 280/280 (100%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280


>g4894847 LIM protein [Mus musculus]
           Length = 280

 Score =  637 bits (1625), Expect = 0.0
 Identities = 278/280 (99%), Positives = 279/280 (99%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSE+FDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSEAFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLA EPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLAGEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280


>g7381058 LIM-only protein FHL3 [Homo sapiens]
```

```
           Length = 280

 Score =  633 bits (1615), Expect = e-180
 Identities = 277/280 (98%), Positives = 277/280 (98%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAP CARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPSCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLA QQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLARQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGC QAGP
Sbjct: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCFQAGP 280


>g30526305 four and a half LIM domains 3 [Sus scrofa]
           Length = 280

 Score =  624 bits (1591), Expect = e-177
 Identities = 271/280 (96%), Positives = 277/280 (98%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSE+FDCAKC+ESLYGRKYIQTD+GPYCVPCYD+TFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSETFDCAKCSESLYGRKYIQTDNGPYCVPCYDSTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSAC ETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACRETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPLGS SFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLGSCSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WHRECLVCTGCQTPLAGQQFTSRD+DPYCVACFGELFAPKCSSCKRPI GLGGGKYVSFE
Sbjct: 181 WHRECLVCTGCQTPLAGQQFTSRDDDPYCVACFGELFAPKCSSCKRPITGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHH+CFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHSCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280


>g5825393 four and half LIM domain protein 3 [Mus musculus]
           Length = 289

 Score =  617 bits (1573), Expect = e-175
 Identities = 270/289 (93%), Positives = 276/289 (95%), Gaps = 9/289 (3%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSE+FDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSEAFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFTCQDSELLCN+CYC+AFSSQCSACGETVMPGSRKLEYGGQ
```

```
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNECYCTAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLCSGCEQPL SRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP
Sbjct: 121 TWHEHCFLCSGCEQPLASRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPI---------VGL 231
           WHRECLVCTGC+TPLAGQ FTSRD+DPYCVACFGELFAPKCSSC RPI          GL
Sbjct: 181 WHRECLVCTGCKTPLAGQHFTSRDDDPYCVACFGELFAPKCSSCNRPITGGSGGAEGAGL 240

Query: 232 GGGKYVSFEDRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           GGGKYVSFEDRHWHH+CFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 GGGKYVSFEDRHWHHSCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 289


>g4416530 skeletal muscle LIM-protein FHL3 [Homo sapiens]
          Length = 280

 Score =  615 bits (1569), Expect = e-175
 Identities = 268/280 (95%), Positives = 272/280 (96%)

Query: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60
           MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH
Sbjct: 1   MSESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRH 60

Query: 61  FHEGCFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120
           FHEGCFRCCRCQRSLADEPFT QDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ
Sbjct: 61  FHEGCFRCCRCQRSLADEPFTRQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQ 120

Query: 121 TWHEHCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQP 180
           TWHEHCFLC GCEQPLGSR FVPDKGAHYCVPCYEN FAPRCARC+KTLTQGG+TYRD P
Sbjct: 121 TWHEHCFLCIGCEQPLGSRPFVPDKGAHYCVPCYENNFAPRCARCTKTLTQGGLTYRDLP 180

Query: 181 WHRECLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240
           WH +CLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE
Sbjct: 181 WHPKCLVCTGCQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFE 240

Query: 241 DRHWHHNCFSCARCSTSLVGQGFVPDGDQVLCQGCSQAGP 280
           DRHWHHNCF+C RCS SLVGQGFVPDGDQVLCQGCSQAGP
Sbjct: 241 DRHWHHNCFTCDRCSNSLVGQGFVPDGDQVLCQGCSQAGP 280


>g8131974 LIM protein FHL3 [Mus musculus]
          Length = 218

 Score =  487 bits (1240), Expect = e-136
 Identities = 210/218 (96%), Positives = 214/218 (97%)

Query: 11  NESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEGCFRCCR 70
           NESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEGCFRCCR
Sbjct: 1   NESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEGCFRCCR 60

Query: 71  CQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQTWHEHCFLCS 130
           CQRSLADEPFTCQDSELLCN+CYC+AFSSQCSACGETVMPGSRKLEYGGQTWHEHCFLCS
Sbjct: 61  CQRSLADEPFTCQDSELLCNECYCTAFSSQCSACGETVMPGSRKLEYGGQTWHEHCFLCS 120

Query: 131 GCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGGVTYRDQPWHRECLVCTG 190
           GCEQPLGSRSFVPDKGAHYCVPCYE    PRCARCSKTLTQGGVTYRDQPWHRECLVCTG
Sbjct: 121 GCEQPLGSRSFVPDKGAHYCVPCYEXNLTPRCARCSKTLTQGGVTYRDQPWHRECLVCTG 180

Query: 191 CQTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPI 228
           C+TPLAGQQFTSRD+DPYCVACFGELFAPKCSSCKRPI
Sbjct: 181 CKTPLAGQQFTSRDDDPYCVACFGELFAPKCSSCKRPI 218
```

```
 Score =  138 bits (345), Expect = 2e-31
 Identities = 64/204 (31%), Positives = 94/204 (45%), Gaps = 6/204 (2%)

Query: 74  SLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQTWHEHCFLCSGCE 133
           SL    +   DS   C  CY + F++ C+ C + +   SR+L Y  + +HE CF C  C+
Sbjct: 3   SLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEGCFRCCRCQ 62

Query: 134 QPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGG--VTYRDQPWHRECLVCTGC 191
           + L    F        C  CY   F+ +C+ C +T+  G   + Y  Q WH  C +C+GC
Sbjct: 63  RSLADEPFTCQDSELLCNECYCTAFSSQCSACGETVMPGSRKLEYGGQTWHEHCFLCSGC 122

Query: 192 QTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFEDRHWHHNCFSC 251
           + PL  + F       YCV C+     P+C+ C + +   G    V++ D+ WH  C  C
Sbjct: 123 EQPLGSRSFVPDKGAHYCVPCYEXNLTPRCARCSKTLTQGG----VTYRDQPWHRECLVC 178

Query: 252 ARCSTSLVGQGFVPDGDQVLCQGC 275
             C T L GQ F    D   C  C
Sbjct: 179 TGCKTPLAGQQFTSRDDDPYCVAC 202


 Score =  119 bits (295), Expect = 1e-25
 Identities = 52/165 (31%), Positives = 79/165 (47%), Gaps = 2/165 (1%)

Query: 5   FDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEG 64
           F C +C  SL    +   DS   C  CY   F++ C+ C + +   SR+L Y  + +HE
Sbjct: 56  FRCCRCQRSLADEPFTCQDSELLCNECYCTAFSSQCSACGETVMPGSRKLEYGGQTWHEH 115

Query: 65  CFRCCRCQRSLADEPFTCQDSELLCNDCYCSAFSSQCSACGETVMPGSRKLEYGGQTWHE 124
           CF C  C++ L    F        C  CY    + +C+ C +T+  G   + Y  Q WH
Sbjct: 116 CFLCSGCEQPLGSRSFVPDKGAHYCVPCYEXNLTPRCARCSKTLTQGG--VTYRDQPWHR 173

Query: 125 HCFLCSGCEQPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTL 169
            C +C+GC+ PL  + F       YCV C+   FAP+C+ C + +
Sbjct: 174 ECLVCTGCKTPLAGQQFTSRDDDPYCVACFGELFAPKCSSCKRPI 218


 Score =  102 bits (252), Expect = 1e-20
 Identities = 46/144 (31%), Positives = 71/144 (48%), Gaps = 4/144 (2%)

Query: 134 QPLGSRSFVPDKGAHYCVPCYENKFAPRCARCSKTLTQGG--VTYRDQPWHRECLVCTGC 191
           + L  R ++      YCVPCY+N FA  CA C + +       + Y D+ +H  C  C  C
Sbjct: 2   ESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLIGHDSRELFYEDRHFHEGCFRCCRC 61

Query: 192 QTPLAGQQFTSRDEDPYCVACFGELFAPKCSSCKRPIVGLGGGKYVSFEDRHWHHNCFSC 251
           Q  LA + FT +D +  C  C+   F+ +CS+C   +  + G + + +  + WH +CF C
Sbjct: 62  QRSLADEPFTCQDSELLCNECYCTAFSSQCSACGETV--MPGSRKLEYGGQTWHEHCFLC 119

Query: 252 ARCSTSLVGQGFVPDGDQVLCQGC 275
           + C   L   FVPD     C  C
Sbjct: 120 SGCEQPLGSRSFVPDKGAHYCVPC 143


 Score = 41.8 bits (96), Expect = 0.025
 Identities = 16/45 (35%), Positives = 23/45 (50%)

Query: 3   ESFDCAKCNESLYGRKYIQTDSGPYCVPCYDNTFANTCAECQQLI 47
           E   C  C   L G+++   D  PYCV C+   FA  C+ C++ I
Sbjct: 174 ECLVCTGCKTPLAGQQFTSRDDDPYCVACFGELFAPKCSSCKRPI 218


  Database: genpept137
    Posted date:  Sep 11, 2003 11:22 AM
  Number of letters in database: 474,463,515
  Number of sequences in database:  1,534,369
```

```
Lambda      K       H
   0.325    0.139    0.497

Gapped
Lambda      K       H
   0.270   0.0470    0.230


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 301682539
Number of Sequences: 1534369
Number of extensions: 13650558
Number of successful extensions: 48959
Number of sequences better than 10.0: 1664
Number of HSP's better than 10.0 without gapping: 615
Number of HSP's successfully gapped in prelim test: 1107
Number of HSP's that attempted gapping in prelim test: 35940
Number of HSP's gapped (non-prelim): 6523
length of query: 280
length of database: 474,463,515
effective HSP length: 46
effective length of query: 234
effective length of database: 403,882,541
effective search space: 94508514594
effective search space used: 94508514594
T: 11
A: 40
X1: 15 ( 7.0 bits)
X2: 38 (14.8 bits)
X3: 64 (24.9 bits)
S1: 40 (21.6 bits)
```

Graphical Viewer...

Submit sequences to: BLAST2 Submit Reset

IncyteGenomics